# UNDERSTANDING THE MOLECULAR MECHANISMS OF TRANSCRIPTIONAL REPRESSION MEDIATED BY KRAB ZINC FINGER PROTEINS AND TRIM28

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# UNDERSTANDING THE MOLECULAR MECHANISMS OF KRAB ZINC FINGER PROTEIN AND TRIM28 MEDIATED TRANSCRIPTIONAL REPRESSION

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KRAB domain Zinc Finger proteins make up the largest family of transcription factors in mammals. Previous studies on a handful of KRAB Zinc Finger proteins have demonstrated that KRAB domains possess the ability to repress transcription, and that this activity is mediated by an interaction with TRIM28. Depleting TRIM28 in mice zygotes results in pre gastrula stage embryonic lethality; however, little is known about how TRIM28 functions with individual KRAB domain proteins to control development. *chatwo*, an ENU-induced mutation, creates a hypomorphic allele of *Trim28*. Interestingly, the phenotype of *chatwo* mutants closely resembles the phenotype of KRAB Zinc Finger protein *Zfp568* mutants. My results demonstrate at the molecular level that TRIM28 physically interacts with ZFP568, and is required for ZFP568 to mediate transcriptional repression. I characterized molecularly the $Trim28^{chatwo}$ allele and found that the *chatwo* mutation impairs the repressive activity of TRIM28, and affects the stability of TRIM28-ZFP568 protein complexes. My results also provide evidence that ZFP568 repressive activity is more severely affected than other KRAB Zinc Finger proteins by TRIM28 depletion, supporting a model in which KRAB Zinc Finger proteins differentially require TRIM28.

By studying mutations in KRAB Zinc Finger protein ZFP568, I found that some KRAB motif amino acid residues are more critical than others to mediate transcriptional repression. My

results also revealed that TRIM28 binding is not sufficient for KRAB domain repressive activity. By analyzing the repressive activity and ability to bind TRIM28 of a wide collection of KRAB domain proteins, I showed that repressive activities of KRAB domain proteins vary broadly amongst different family members, and importantly, the repressive activities of KRAB Zinc Finger proteins do not correlate with their ability to interact with TRIM28. Overall, this study provides novel contributions to the current understanding of the transcriptional roles of KRAB Zinc Finger proteins.

# BIOGRAPHICAL SKETCH

Kristin Murphy, the oldest of two daughters, was born in 1984 in Victoria, Texas. Kristin spent the majority of her childhood in Fairfield, California, and later attended high school in Wilmington, Delaware. She attended college at the University of Delaware, and while studying biology there, she worked part time at the USDA Applied Ecology and Beneficial Insects laboratory. Kristin was interested in both ecology and genetics, but became particularly excited about pursuing a career in genetic research after completing two courses with Dr. Patricia DeLeon. After receiving her B.A. in Biological Sciences, Kristin worked as a laboratory technician in the laboratory of Dr. David Strayer at Thomas Jefferson University in Philadelphia, Pennsylvania. Here, she researched gene-therapy based immunization against Botulinum neurotoxin. Kristin began graduate school at Cornell University in the field of Genetics and Development in 2007. She joined the laboratory of Dr. Maria J. Garcia-Garcia in 2008.

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LIST OF ABBREVIATIONS

KRAB: Kruppel-associated box

DNA: Deoxyribonucleic acid

C2H2: Cys2His2

SCAN: SRE-ZBP, CTfin51, AW-1 and Number 18 cDNA

TRIM28: tripartite motif containing 28

KAP-1: KRAB-associated protein 1

KRIP-1: KRAB-interacting protein 1

TIF1β: transcription intermediary factor 1-beta

ZFP568: zinc finger protein 568

RSL1: regulator of sex-limited protein 1

Slp: sex-limited protein

TRIM: tripartite motif

RBCC: ring b-box coiled-coil

PHD: plant homeodomain

HP1: heterochromatin protein 1

TRIM24: tripartite motif containing 24

TRIM33: tripartite motif containing 33

CHD3: chromodomain helicase DNA binding protein 3

NuRD: nucleosome remodeling and deacetylase

ATPase: adenosine triphosphatase

HDAC: histone deacetylase

H3K9: histone 3 lysine 9

SETDB1: SET domain, bifurcated 1

PolII: polymerase II

SUMO: small ubiquitin-like modifier

UBC9: ubiquitin-conjugating enzyme E2I

ATM: ataxia telangiectasia mutated

E: embryonic day

ES: embryonic stem

OCT4: octamer-binding protein 4

ZFP809: zinc finger protein 809

PBS: primer binding site

ZFP57: zinc finger protein 57

ChIP-chip: chromatin immunoprecipitation on microarray

ChIP-seq: chromatin immunoprecipitation sequencing

ZNF274: zinc finger protein 274

SMAD4: mothers against decapentaplegic homolog 4

PHB2: prohibitin 2

DBD: DNA-binding domain

AD: activation domain

*T*: brachyury

CPRG: chlorophenol red-β-D-galactopyranoside

siRNA: small interfering RNA

ZFP110: zinc finger protein 110

ZNF568: zinc finger protein 568

ZNF28: zinc finger protein 28

ZFP69: zinc finger protein 69

ZFP617: zinc finger protein 617

ZFP446: zinc finger protein 446

ZFP496: zinc finger protein 496

ZFP13: zinc finger protein 13

RNAi: RNA interference

SMAD: mothers against decapentaplegic homolog

ZF: zinc finger

# CHAPTER 1

# INTRODUCTION

Transcription factors contain deoxyribonucleic acid (DNA) binding elements that recognize specific genomic sites, and possess the ability to regulate gene expression (Brivanlou and Darnell, 2002) A number of mechanisms exist by which transcription factors can precisely control the spatial and temporal patterns of gene expression. As a consequence, transcription factors and are widely utilized by eukaryotes during development for the initiation of specific events at the correct time in the correct subset of cells (Spitz and Furlong, 2012).

## KRAB ZINC FINGER PROTEINS

### KRAB Zinc Finger protein evolution

Kruppel-associated box (KRAB) Zinc Finger proteins comprise the largest family of transcription factors in mammals (Urrutia, 2003). There are over 400 family members encoded in each the mouse and human genomes, and they make up more than one third of the Zinc Finger containing genes in those species (Margolin et al., 1994; Urrutia, 2003). Despite their large numbers in mammals, only a small number of KRAB Zinc Finger genes have been identified in frogs, and none have been discovered in fish (Fig. 1.1A) (Urrutia, 2003). In fact, KRAB domains are only present in tetrapod vertebrate organisms (Urrutia, 2003; Emerson and Thomas, 2009). KRAB domain proteins have not only evolved relatively recently, but they have been shown to be under positive selection (Bustamante et al., 2005; Lorenz et al., 2010). Such a recent evolutionary expansion suggests that they have important functions in processes specific to tetrapods (Urrutia, 2003).

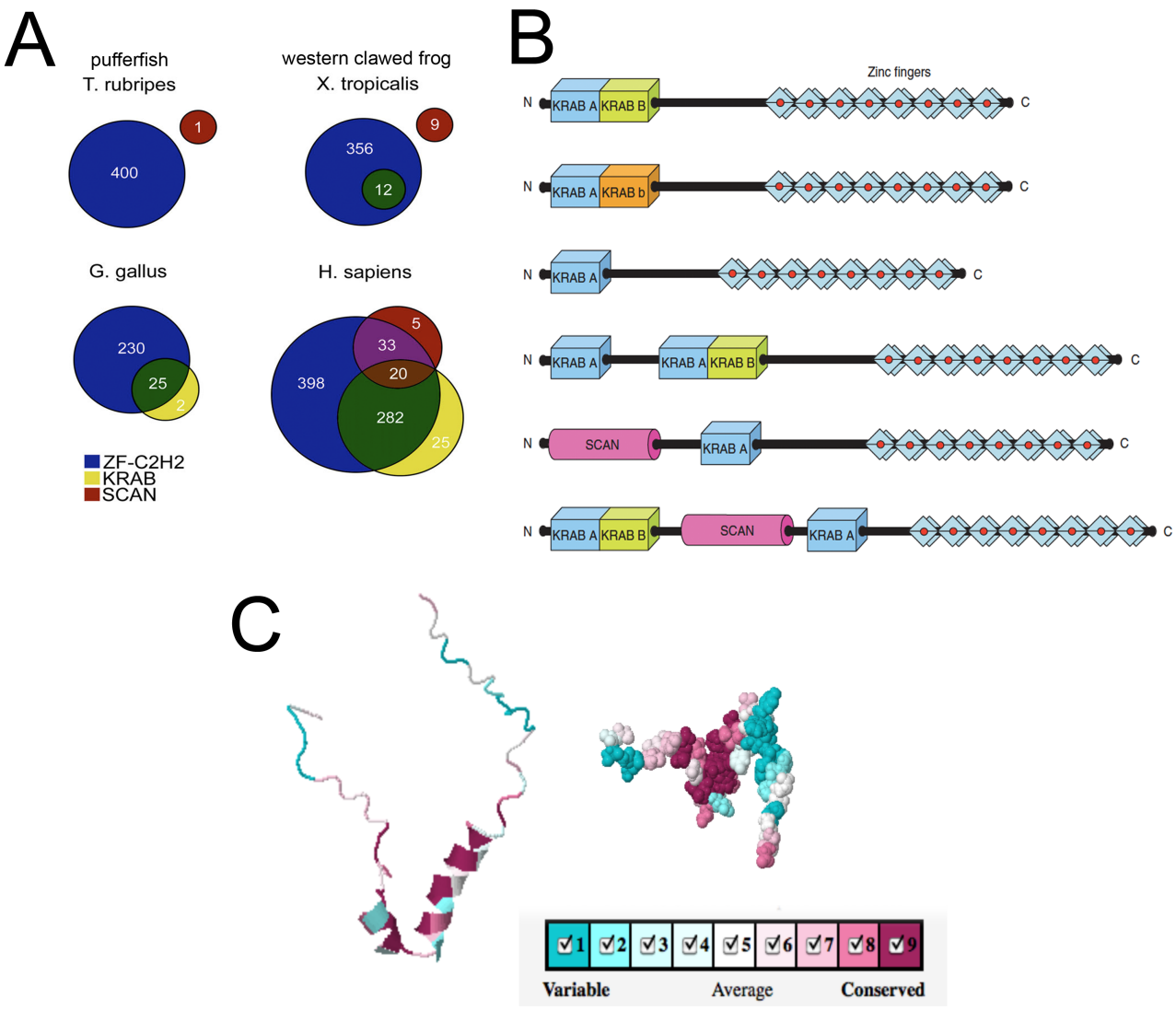



**Figure 1.1. KRAB Zinc Finger protein domain organization, evolution, and structure.** (A) Diagram illustrating the numbers of KRAB domain containing proteins in species *T. rubripes, X. tropicalis, G. gallus, and H. sapiens*. Adapted from Thomas and Emerson, 2009. (B) Scheme showing domain organization of KRAB Zinc Finger proteins. Proteins may contain KRAB A box, KRAB A box and KRAB B box, KRAB A box and KRAB b box, multiple KRAB domain combinations, or KRAB and SCAN domain combinations, and a variable number Zinc Finger domains. Adapted from Urrutia, 2003. (C) KRAB domain structure generated with CONSURF (http://consurf.tau.ac.il/credits.php) using compilation of all mouse KRAB domain amino acid sequences and the predicted KRAB domain structure (Madej et al., 2012). Turquoise colors/ lower numbers represent less conserved residues and dark pink colors/higher numbers represent more conserved residues.

KRAB Zinc Finger proteins contain one or more KRAB domains at their amino terminal end, and a variable number of $Cys_2His_2$ (C2H2) Zinc Finger domains at their carboxyl terminal end (Margolin et al., 1994). There are several possible KRAB domain configurations. Some proteins contain only the most conserved 45 amino acid KRAB A box, while some contain a KRAB A box followed by a conserved KRAB B box, and others contain a KRAB A box followed by a more degenerate KRAB b box. A proportion of KRAB Zinc Finger proteins contain a SRE-ZBP, CTfin51, AW-1 and Number 18 cDNA (SCAN) domain in addition to KRAB domain(s) at their amino terminus (Fig. 1.1B) (Urrutia, 2003). KRAB Zinc Finger genes are organized in clusters throughout the genome, suggesting that their evolutionary expansion occurred through gene and chromosome segmental duplications (Urrutia, 2003; Hamilton et al., 2006). Additionally, they possess a conserved exon configuration, in which each KRAB domain is encoded in a single exon and all Zinc Finger domains are encoded together in another single exon, providing a regulatory mechanism for alternative splicing in order to include or exclude specific domains (Bellefroid et al., 1993; Vissing et al., 1995; Urrutia, 2003).

**KRAB Zinc Finger protein molecular function**

*In vitro* studies published nearly 20 years ago on a number of KRAB Zinc Finger proteins determined that KRAB domains possess repressive activity when artificially bound to reporter genes. (Margolin et al., 1994; Pengue et al., 1994; Witzgall et al., 1994; Vissing et al., 1995). The KRAB A box is required for transcriptional repression, while the KRAB B box can enhance repressive activity (Vissing et al., 1995). KRAB domain repression is dependent on local DNA binding and does not affect global transcription levels (Margolin et al., 1994; Witzgall et al.,

1994). The mechanism by which KRAB domains repress transcription is by recruiting co-factors that modify the chromatin environment. To date, over 20 human and mouse KRAB domains have been reported to possess repressive activity by reporter assays (Table 1.1). Thus, transcriptional repression is considered the primary function for the KRAB Zinc Finger protein family (Urrutia, 2003).

The repressive activities of KRAB domains are presumed to depend on an interaction with the protein tripartite motif-containing 28 (TRIM28), also named KRAB associated protein 1 (KAP-1), KRAB interacting protein 1 (KRIP-1), and transcription intermediary factor 1-beta (TIF1β). TRIM28 was isolated as a binding partner for several KRAB domain proteins, and has since been shown to interact with many more KRAB domain proteins (Moosmann et al., 1996, Kim et al., 1996). Purified TRIM28 bound to a KRAB domain is more resistant to protease digestion than the KRAB domain alone, suggesting that the TRIM28 interaction stabilizes KRAB Zinc Finger proteins (Peng et al., 2007). Reporter assays performed with a number of KRAB Zinc Finger proteins have demonstrated that KRAB repressive activity is mediated by TRIM28 protein levels (Friedman et al., 1996; Sripathy et al., 2006), and that TRIM28 is required for repressive activity (Sripathy et al., 2006). KRAB domain mutations that disrupt the interaction with TRIM28 correlate with a loss of KRAB repressive activity (Kim et al., 1996; Moosmann et al., 1996; Ryan et al., 1999). Consequently, TRIM28 has been speculated to be a universal co-repressor for all KRAB Zinc Finger proteins (Urrutia, 2003).

Zinc Finger domains are known to possess sequence specific DNA binding affinities (Zheng et al., 2000; Gebelein and Urrutia, 2001; Peng et al., 2002b). It has been demonstrated *in vitro* that one C2H2 Zinc Finger domain can interact with three nucleotides, and that the amino

acid sequence of a Zinc Finger domain can dictate which nucleotides are bound (Miller et al., 2007). Several KRAB Zinc Finger protein consensus binding sequences have been identified using *in vitro* oligonucleotide selection assays (Zheng et al., 2000; Gebelein and Urrutia, 2001), and a few binding sites have been confirmed using chromatin immunoprecipitation (ChIP) *in vivo* (Peng et al., 2002b; Wolf and Goff, 2008; Quenneville et al., 2011; Krebs et al., 2012). Of these, consensus binding sites are considerably shorter than the predicted length using a model in which all Zinc Finger domains bind DNA. This suggests that not all Zinc Finger domains are utilized simultaneously (Jing et al., 2004).

While crystal structures of full-length KRAB domain proteins are not yet available, several studies provide insight about the molecular structure of the KRAB motif. The conserved amino acids within the 45 amino acid KRAB A box are predicted to fold into two amphipathic alpha helices (Bellefroid et al., 1993). Each alpha helix is predicted to be configured with the most conserved and amphipathic amino acids on one face of the helix pointing toward the second alpha helix, providing an ideal pocket for an interaction with a protein such as TRIM28 (Fig. 1.1C) (Peng et al., 2007). In addition to the KRAB domain predicted structure, a crystal structure of two adjacent zinc finger domains of one KRAB Zinc Finger protein, ZFP57, has recently been solved in a complex with DNA. This study found that ZFP57 specifically binds to a methylated DNA sequence, supporting the proposition that KRAB Zinc Finger proteins interact with specific DNA elements (Liu et al., 2012).

Together, the *in vitro* evidence for KRAB domain repressive activity along with sequence specific DNA binding capabilities has led to a model in which the KRAB Zinc Finger protein family functions to repress transcription at specific target DNA sequences. The large number of

expanded mammalian KRAB Zinc Finger proteins has been proposed to provide a mechanism for tissue and temporal specific control of transcription (Urrutia, 2003).

**Table 1.1: KRAB Zinc Finger protein functions.** Table indicating functions identified for individual mouse and human KRAB Zinc Finger gene and corresponding publications. Genes with Zfp annotation correspond to mouse, and genes with ZNF annotation correspond to human.

**Roles of individual KRAB Zinc Finger proteins**

While the biological functions of the vast majority of KRAB Zinc Finger proteins encoded in the mouse and human genomes remain elusive, functions of a number of family members have been uncovered. Their roles include regulation of important biological processes such as ES cell pluripotency, retroviral silencing, DNA damage repair, carcinoma suppression, imprinting, embryonic morphogenesis, mammary gland development, and sex-specific pubertal timing (Table 1.1) (Huang et al., 2007; García-García et al., 2008; Li et al., 2008; Wolf and Goff, 2008; Tian et al., 2009; Cheng et al., 2010; Wang et al., 2010; Shibata and García-García, 2011; Yuan et al., 2012; Krebs et al., 2012; Oliver et al., 2012). Many of these functions are specific to higher organisms, supporting the idea that KRAB Zinc Finger genes expanded recently in evolution in order to fulfill new functions. While some KRAB Zinc Finger proteins are ubiquitously expressed, others have tissue or stage-specific expression profiles (Bellefroid et al., 1993; Witzgall et al., 1994; García-García et al., 2008). Furthermore, there does not seem to be functional redundancy among KRAB Zinc Finger proteins identified thus far (Table 1.1).

In support of the *in vitro* evidence for TRIM28 functioning as a co-repressor with KRAB Zinc Finger proteins, several studies have shown that TRIM28 interacts with and contributes to the functions of individual KRAB Zinc Finger proteins *in vivo*. For example, zinc finger protein 809 (ZFP809) binds to and functions to represses retroviral elements with TRIM28. Zinc finger

| gene | citation | function |
|---|---|---|
| *Kox1, Znf141, Znf133, Znf140* | (Margolin et al., 1994) | Repressive activity |
| *Kid1, Znf2* | (Witzgall et al., 1994) | Repressive activity |
| *Znf10, Znf133, Znf140. Znf141, Znf136* | (Vissing et al., 1995) | Repressive activity |
| *Znf350/Zbrk1* | (Zheng et al., 2000; Peng et al., 2002b; Hallen et al., 2011) | Interacts with BRCA1, binds GADD45 intron, interacts with ATXN, regulates SCA2 transcription |
| *mZnf8* | (Jiao et al., 2002) | Repressive activity, interacts with SMAD1 |
| *Znf74* | (Germain-Desprez et al., 2003) | Interacts with TRIM proteins in nuclear matrix |
| *Znf333* | (Jing et al., 2004) | Repressive activity, consensus binding sequence identified |
| *Krab-O* | (Oh et al., 2005; Oh and Lau, 2006; Peng et al., 2009) | Interacts with SRY and TRIM28, mediates SRY |
| *Zfp224* | (Medugno et al., 2005; Cesaro et al., 2009) | Repressive activity, binds to AdlA-NRE motif, binds PRMT5 |
| *Tipuh1* | (Silva et al., 2006) | Interacts with TRIM28 and hnRNPs, upregulated in hepatocellular carcinoma |
| *Znf23* | (Huang et al., 2007) | Frequently altered in solid tumors, supresses cell growth and induces cell arrest |
| *Zfp496* | (Mysliwiec et al., 2007; Kahns et al., 2010; Losson and Nielsen, 2010) | Different isoforms activate/repress transcription, interacts with JUMONJI and TRIM28 |
| *Znf307* | (Li et al., 2007) | Repressive activity, represses p53 and p21 at protein level |
| *Znf300* | (Qiu et al., 2008; Xu et al., 2010; Xue et al., 2010) | Repressive activity, consensus binding sequence identified, can activate IL-2Rbeta, upregulated in AML/CML, expression mediated by PU.1 |
| *Zfp568* | (García-García et al., 2008; Shibata and García-García, 2011; Shibata et al., 2011) | Repressive activity, interacts with TRIM28, controls embryonic and extraembryonic morphogenesis |
| *Zfp57* | (Li et al., 2008; Quenneville et al., 2011; Zuo et al., 2012) | Downregulated upon ES cell differentiation, interacts with TRIM28, mouse partial lethality in homozygosity with maternal effect, controls imprinted gene expression and maintenance of methylation, consensus binding sequence identified |
| *Zfp647* | (Briers et al., 2009) | Localizes to sub-cellular foci with TRIM28 and HP1 |
| *Zfp809* | (Wolf and Goff, 2009) | Binds to PBS of MLV, binds TRIM28, silences retroviral expression in ES cells |

| | | |
|---|---|---|
| *Apak* | (Tian et al., 2009; Wang et al., 2010; Yuan et al., 2012) | Interacts with p53, reduces p53 activity and Bax expression, interacts with ATM in TRIM28 dependent manner, phosphorylated by ATM, consensus binding sequence identified |
| *Zfp69* | (Scherneck et al., 2009) | Candidate for accelerated diabetes |
| *Znf426* | (Yang et al., 2009) | Represses viral transactivator, consensus binding sequence identified |
| *Znf263* | (Frietze et al., 2010a) | *in vivo* binding sites identified, does not interact with TRIM28 |
| *Znf552* | (Deng et al., 2010) | Repressive activity, represses AP-1 and SRE |
| *Znf424* | (Wang et al., 2010) | Represses NFAT and p21 |
| *Rsl1* | (Krebs and Robins, 2010; Krebs et al., 2012) | Regulates expression of Slp, controls pubertal timing, *in vivo* binding sequence identified |
| *Znf382* | (Cheng et al., 2010) | Represses oncogenes, disrupted in multiple carcinoma lines, induces apoptosis of tumor cells |
| *Znf274* | (Frietze et al., 2010b) | Repressive activity, expressed in a number of cancer cell lines, *in vivo* binding sites identified, interacts with and binds same sites as TRIM28 and SETDB1, binds to 3' ends of some KRAB and SCAN containing genes |
| *ZkSCAN3* | (Yang et al., 2011) | Upregulated in myeloma cell lines, binds cyclin D2 promoter and induces its expression |
| *Znf425* | (Wang et al., 2011) | Represses AP-1, SRE, and SRF mediated transcriptional activity |
| *Znf764/Paris* | (Shin et al., 2011) | Interacts with PARKIN, PARKIN ubiquitinated by PARKIN, consensus binding sequence identified, represses PGC-1alpha promoter activity |
| *Zifcat* | (Gu et al., 2011) | Repressive activity, interacts with gamma-catenin |
| *Znf431* | (He et al., 2011) | Repressive activity, interacts with HDAC2, binds promoter of PATCHED1, represses Patched1 *in vivo* in *xenopus* and mouse embryos |
| *Znf268* | (Zeng et al., 2012) | Downregulated during erythroid differentiation, represses tumor growth, repressed by GATA1 |
| *Zfp157* | (Oliver et al., 2012) | Upregulated in Stat6 null tissue, repressed by STAT6 and GATA3, controls rate of mammary development |
| *Znf545* | (Cheng et al., 2012) | Represses AP1 and NFkappaB, downregulated in carcinomas, sequestered in nucleoli, inhibits ribosomal DNA |

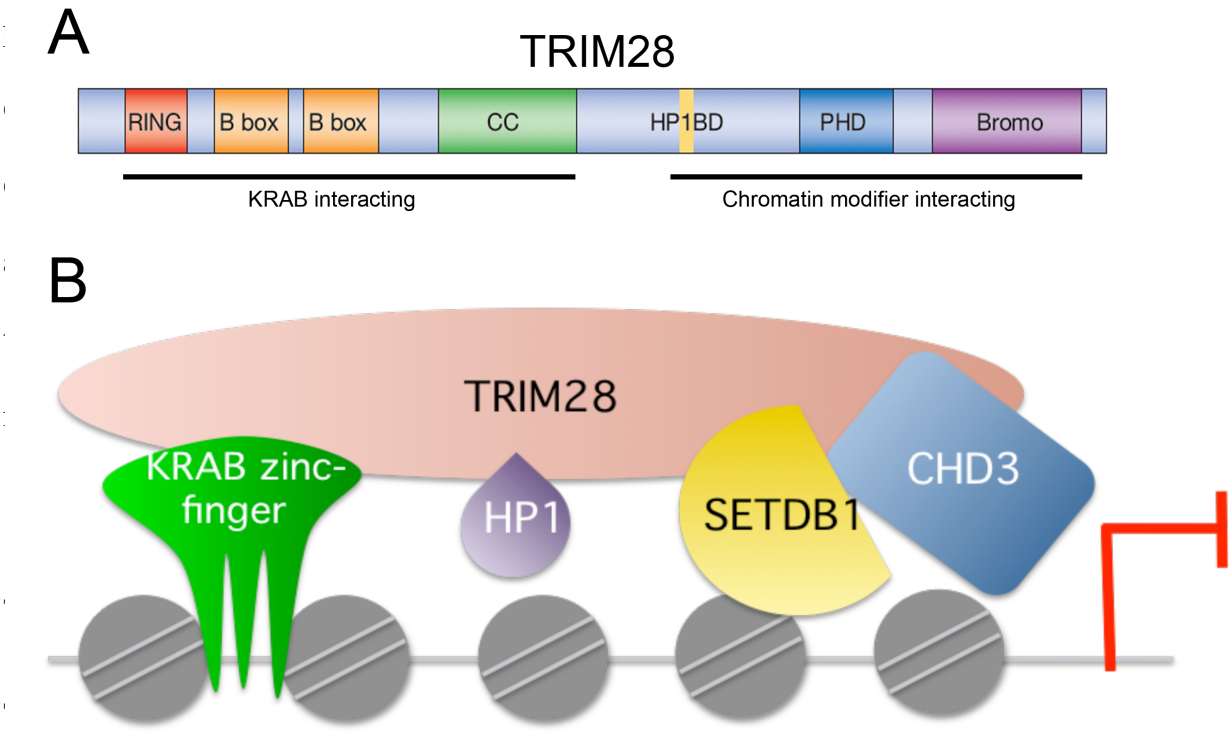



**Figure 1.2. TRIM28 domain model for transcriptional repression.** (A) Schematic of domain organization of TRIM28 protein. Adapted from Ziv et al., 2006. (B) Cartoon showing model in which TRIM28 functions as molecular scaffold by interacting with KRAB Zinc Finger proteins provides sequence specificity and chromatin modifying proteins mediate transcriptional repression (Friedman et al., 1996; Moosmann et al., 1996). When artificially targeted to

domain, at its amino terminus. At its carboxyl terminus, TRIM28 contains a plant homeodomain (PHD)-bromodomain, and in between these terminal domains lies a heterochromatin protein 1 (HP1) binding motif (Figure 1.2, A) (Friedman et al., 1996; Le Douarin et al., 1996; Ryan et al., 1999; Lechner et al., 2000). The interaction with KRAB domains occurs through the RBCC domain of TRIM28 (Friedman et al., 1996;

DNA, TRIM28 possesses inherent repressive activity (Friedman et al., 1996; Moosmann et al., 1996; Ryan et al., 1999), which depends on both the HP1 binding motif and the PHD-bromodomain (Schultz et al., 2001). Biochemical studies have revealed that TRIM28 exists in homo-oligomeric states (Peng et al., 2000a, 2000b, 2002a). Similar to other RBCC domain containing proteins, TRIM28 oligomerization occurs through its RBCC domain (Saurin et al., 1996). TRIM28 primarily exists in trimeric forms, which are particularly stabilized when bound

to a KRAB domain (Peng et al., 2000a).

The closest family member to TRIM28 in mouse is tripartite motif-containing 24 (TRIM24). TRIM24 binds retinoic acid receptor genes, and although TRIM28 is highly homologous, it does not share this function (Peng et al., 2002a; Khetchoumian et al., 2007). Whereas TRIM28 can repress transcription, TRIM24 cannot (Schultz et al., 2001). Furthermore, TRIM28 and TRIM24 do not hetero-oligomerize (Peng et al., 2002a), suggesting that the functions of these proteins have diverged significantly.

TRIM28 also shares similarity to other PHD-bromodomain containing proteins. bromodomains in other proteins typically recognize acetylated histones, and PHD domains often recognize trimethylated histones (Baker et al., 2008; Filippakopoulos and Knapp, 2012). However, there is no evidence that the PHD-bromodomain of TRIM28 binds modified histones (Peng et al., 2002a). Furthremore, the repressive activity of TRIM28 is not maintained if the PHD or bromodomain is swapped with that of another protein (Schultz et al., 2001) A crystal structure of the PHD-bromodomain of TRIM28 has revealed that the bromodomain lies directly adjacent to the PHD domain, suggesting that the configuration of the PHD-bromodomain of TRIM28 is distinct from that of other proteins (Peng and Wysocka, 2008; Zeng et al., 2008). In general, TRIM28 shares many conserved domains with other proteins, but uniquely functions in mediating KRAB domain repression.

Several chromatin-modifying proteins have been described to bind TRIM28, suggesting that TRIM28 represses transcription through epigenetic mechanisms. Isoforms α, β, and γ HP1 have all been shown to interact with TRIM28 through its HP1 binding motif to form complexes that include KRAB domains and DNA (Ryan et al., 1999). In support of this, TRIM28 co-

localizes with HP1 in heterochromatin puncti in nuclei (Nielsen et al., 1999; Ryan et al., 1999). Chromatin helicase DNA binding protein 3 (CHD3), a member of the nucleosome remodeling and deacetylase (NuRD) complex which also contains adenosine triphosphatase (ATPase) chromatin remodelers and histone deacetylases (HDAC) (Schultz et al., 2001; Denslow and Wade, 2007), binds TRIM28 cooperatively through its PHD-bromodomains. In a similar manner, the histone 3 lysine 9 (H3K9) methyltransferase SET domain bifurcated 1 (SETDB1) also binds TRIM28 through its PHD-bromodomain (Schultz et al., 2001, 2002).

HP1, CHD3, and SETDB1 all regulate the repressive activities of both TRIM28 and KRAB domains *in vitro*, and the repressive roles of HP1 and CHD3 are at least partially dependent on HDACs (Nielsen et al., 1999; Ryan et al., 1999; Schultz et al., 2001). H3K9me3, the modification catalyzed by SETDB1, is associated with silenced genes, and is required for HP1 recruitment to chromatin (Bannister et al., 2001). TRIM28, HP1, CHD3, and SETDB1 have been shown to associate with the promoter regions of silenced genes. At these sites, H3K9 trimethylation is increased, H3 acetylation is decreased, and RNA Polymerase II (PolII) binding is reduced, indicative of heterochromatin formation (Sripathy et al., 2006). These lines of evidence have lead to a model in which TRIM28 functions as a scaffold, localizing specific chromatin-modifying proteins to DNA, and causing local transcriptional silencing (Figure 1.2B) (Schultz et al., 2002).

**Post-translational regulation of TRIM28**

Small ubiquitin related modifier (SUMO) modifications regulate TRIM28 function post-tranlationally, ultimately promoting its repressive activity. The PHD domain of TRIM28 binds to ubiquitin conjugating enzyme E2I (UBC9) and functions as an intramolecular SUMO ligase to SUMOylate its adjacent bromodomain (Ivanov et al., 2007). TRIM28 is a target of SUMOylation at six different lysine residues (Ivanov et al., 2007; Lee et al., 2007; Mascle et al., 2007). Importantly, mutations that disrupt TRIM28 SUMO acceptor sites also affect its ability to repress transcription (Mascle et al., 2007). More specifically, SUMOylation of TRIM28 is required for binding to chromatin modifiers SETDB1 and CHD3, homo-oligomerization, and HDAC mediated repressive activity (Ivanov et al., 2007; Lee et al., 2007; Mascle et al., 2007). Furthermore, KRAB domains enhance TRIM28 SUMOylation, suggesting that the association of KRAB Zinc Finger proteins with TRIM28 positively regulates repressive activity of the entire complex (Mascle et al., 2007).

In addition to SUMOylation, TRIM28 is subject to post-transcriptional phosphorylation modifications. Phospho-TRIM28 species were first identified as a target of the phosphokinase C family in response to DNA damage (White et al., 2006; Ziv et al., 2006). Serine residue 473 (Ser473) phosphorylation of TRIM28 is regulated with the cell cycle, specifically prominent during S and M phases (Chang et al., 2008). Located near the HP1 binding domain of TRIM28, Ser473 phosphorylation inhibits HP1beta binding. In addition, it prevents TRIM28 auto-SUMOylation from occurring, consequently impeding CHD3 binding (Goodarzi et al., 2011). In response to DNA double strand breaks, Ser824 is dephosphorylated in an ataxia telangiectasia mutate (ATM) dependent manner, ultimately promoting chromatin relaxation and facilitating

DNA repair (Ziv et al., 2006; Goodarzi et al., 2010; Noon et al., 2010). In this way, both phosphorylation and SUMOylation post-translational modifications function cooperatively to regulate TRIM28 downstream effects.

### *In vivo* roles of TRIM28

TRIM28 is required for post-gastrulation development in mouse. Homozygous deletion mutants arrest development at embryonic day (E) 5.5, shortly after implantation (Cammas et al., 2000). This early embryonic arrest phenotype is consistent with TRIM28's proposed role as a universal co-repressor for all KRAB Zinc Finger proteins. Deletion of TRIM28 in a tissue-specific manner has demonstrated broad requirements for TRIM28 during late development and in adult tissues. For example, TRIM28 is essential in male germ cells for maintenance of spermatogenesis (Weber et al., 2002), and TRIM28 deletion in the mouse forebrain leads to behavioral disabilities in response to stress (Jakobsson et al., 2008).

Several lines of evidence also implicate TRIM28 in the maintenance of embryonic stem (ES) cell pluripotency, as well as cellular differentiation. The HP1 interaction domain of TRIM28 is necessary for terminal differentiation of primitive endoderm-like cells into visceral endoderm-like cells (Cammas et al., 2004). TRIM28 was also identified in a screen as a required factor for maintenance of ES cell self-renewal (Hu et al., 2009). In fact, TRIM28 Ser824 phosphorylation is required in pluripotent cells for TRIM28 to interact with octamer binding protein 4 (OCT4) and association with euchromatin. Upon differentiation, however, Ser824 phosphorylation is reduced and TRIM28 promotes heterochromatinization (Seki et al., 2010).

## TRIM28 AND KRAB ZINC FINGER PROTEIN GENOMIC TARGETS

Just as the functions of most individual KRAB Zinc Finger proteins are still unknown, the genomic targets of TRIM28-KRAB Zinc Finger complexes are as well. While consensus binding sequences have been identified for over 15 mouse and human KRAB Zinc Finger proteins, these studies do not confirm the functional significance of their binding (Table 1.1). A number of comprehensive studies, however, have shed some light on mechanisms for KRAB Zinc Finger DNA binding. For example, ZFP809 has been shown to bind retroviral primer binding site (PBS) DNA elements. ZFP809 is expressed in ES cells, where it recruits TRIM28 to the PBS in order to silence the expression of retroviral elements. ZFP809 binding is highly specific to the exact PBS sequence, as only one nucleotide substitution can ablate its binding affinity (Wolf and Goff, 2009). TRIM28 and ZFP57 have recently emerged as regulators of genomic imprinting. TRIM28 was found to bind to the promoter and regulate the expression of MEST and other imprinted genes (Riclet et al., 2009; Quenneville et al., 2011), and ZFP57 regulates the expression of several imprinted loci (Li et al., 2008; Quenneville et al., 2011). Recently, a hexanucleotide sequence has been identified within imprinted loci that is recognized by ZFP57 (Quenneville et al., 2011).Thus, ZFP57, like ZFP809, targets a specific DNA sequence to regulate the expression of a defined class of genes.

In addition to the identified binding sites of individual KRAB Zinc Finger proteins, several whole-genome analysis studies have revealed additional targets for TRIM28 and KRAB domain proteins. Chromatin immunoprecipitation on microarray (ChIP-chip) experiments in ES cells identified TRIM28 occupancy with other regulators of pluripotency at gene promoters,

corroborating its role in controlling pluripotency and differentiation (Hu et al., 2009). Microarray and chromatin immunoprecipitation sequencing (ChIP-seq) experiments have also shown that TRIM28 binds to genomic sites largely shared by both SETDB1 and H3K9me3, supporting a functional role for the TRIM28 repressive complex (O'Geen et al., 2007; Frietze et al., 2010b; Iyengar and Farnham, 2011; Iyengar et al., 2011). While TRIM28 does bind to the promoters of some genes, a high proportion of TRIM28 binding sites actually occur in the 3' ends of Zinc Finger genes. It is unclear at this point whether TRIM28 binding to these Zinc Finger genes promotes their silencing or fulfills a separate function (Iyengar and Farnham, 2011). Furthermore, it remains to be seen which KRAB Zinc Finger or other DNA binding proteins are directing TRIM28 to different genomic locations. Only two KRAB Zinc Finger proteins have been analyzed for genome-wide occupancy to date. One of these, zinc finger protein 274 (ZNF274), does in fact bind to the 3' ends of Zinc Finger genes (Frietze et al., 2010b), suggesting that KRAB Zinc Finger proteins may utilize autoregulation by recruiting TRIM28 to KRAB Zinc Finger genes.

One caveat to whole-genome analyses is that they have been performed in only a couple of cell types, mostly cancer cell lines (Frietze et al., 2010a, 2010b). Because KRAB Zinc Finger proteins have specific and varied expression patterns (Urrutia, 2003), their genomic localization may be better elucidated with a genome-wide studies in a profile of different tissues and developmental times. Several complications, however, have hindered the study of KRAB Zinc Finger proteins in non-reporter systems. First, antibodies that specifically recognize a single KRAB Zinc Finger protein are difficult to generate because of the high degree of similarity between family members (Urrutia, 2003; Frietze et al., 2010a). Second, attempts at generating

stable cell lines with tagged KRAB Zinc Finger proteins have often resulted in truncated gene incorporation for unknown reasons (Wolf and Goff, 2009; Iyengar and Farnham, 2011). Therefore, our knowledge about *in vivo* KRAB Zinc Finger genome-wide occupancy remains limited.

It is clear that additional research about KRAB Zinc Finger proteins and TRIM28 will contribute significantly to our knowledge about transcriptional regulation and mammalian development. While the most fundamental KRAB Zinc Finger binding partners and molecular functions have been elucidated for a handful of family members, it is critical to gain a more comprehensive understanding about the similarities and divergences between the molecular functions of different KRAB Zinc finger proteins in different cell types and developmental times.

## ORGANIZATION OF DISSERTATION

The goal of this dissertation is to determine the molecular functions of ZFP568 and TRIM28 in controlling morphogenesis during mouse embryogenesis, as well as to gain novel insights about the relationship between KRAB domain repressive activity and TRIM28.

In Chapter 2, I present my research on the molecular consequences of a mutation in *Trim28* on the functions of TRIM28 and KRAB Zinc Finger proteins. I show that the repressive functions of KRAB Zinc Finger protein ZFP568 are severely disrupted by the mutation, and I provide evidence that KRAB Zinc Finger proteins differentially require TRIM28.

In Chapter 3, I present a study about KRAB domain protein transcriptional repression. Analysis of a mutation in KRAB Zinc Finger protein ZFP568 demonstrates that an interaction

with TRIM28 is not sufficient for repressive activity. I identify multiple mouse and human KRAB domains lacking efficient repressive activities, and determine that KRAB domain mediated transcriptional repression is not always correlated with TRIM28 binding.

In Chapter 4, I review the conclusions and discuss the implications of my dissertation research. I propose mechanisms to explain my findings, and provide ideas for possible future experiments for the study of KRAB Zinc Finger proteins.

In Appendix A, I describe results from an unbiased yeast two-hybrid screen for proteins that interact with ZFP568. I show additional data from candidate driven approaches, which suggest that the KRAB domains of ZFP568 interact with mothers against DPP homolog 4 (SMAD 4).

In Appendix B, I present follow-up studies on the interaction between ZFP568 and Prohibitin 2 (PHB2) identified in the yeast two-hybrid screen discussed in Appendix A. My results suggest that PHB2 interacts with TRIM28 in addition to ZFP568, and bring about the possibility that PHB2 mediates the repressive activity of ZFP568.

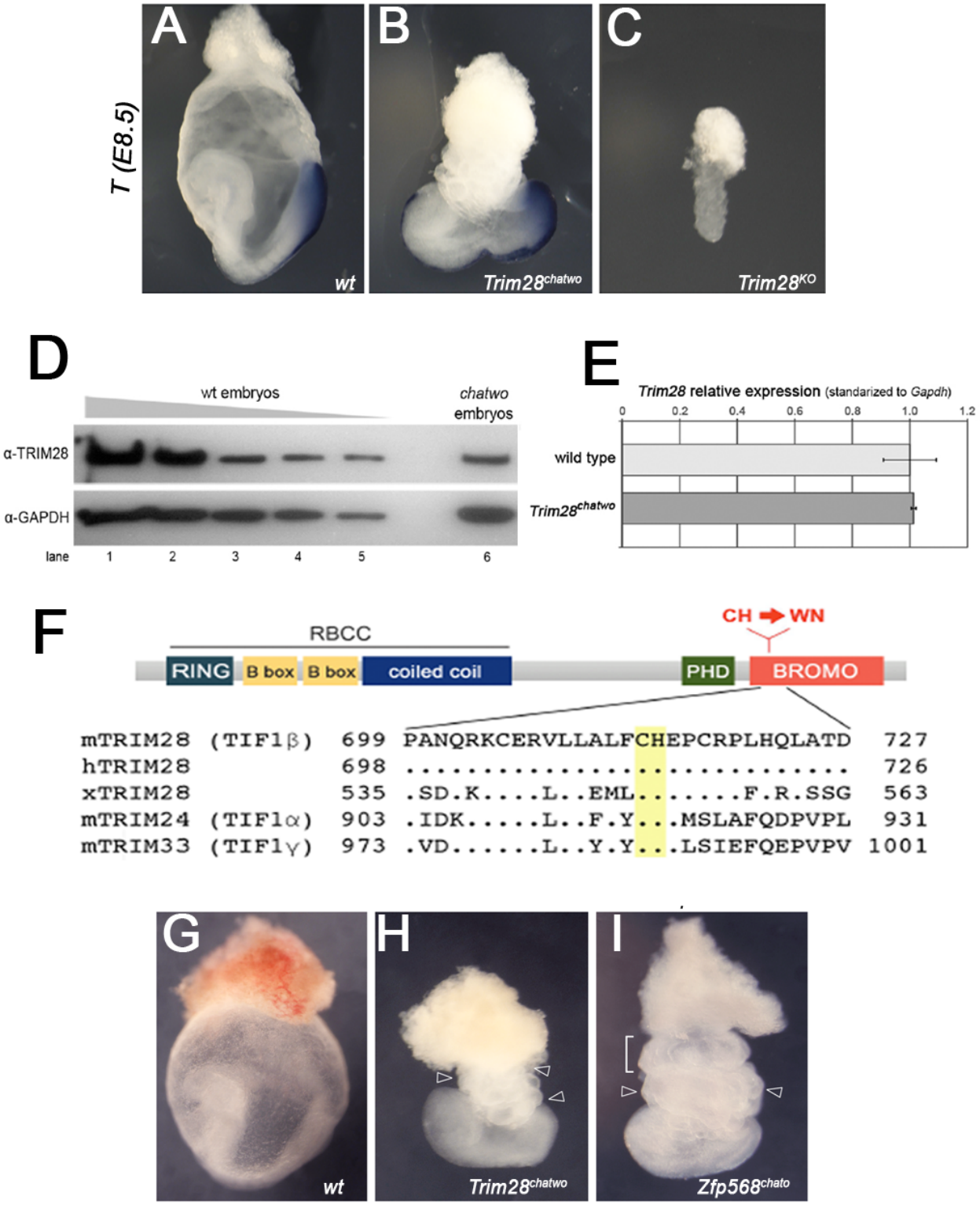
# CHAPTER 2

# THE TRANSCRIPTIONAL CO-REPRESSOR TRIM28 IS DIFFERENTIALLY REQUIRED BY DISTINCT KRAB DOMAIN PROTEINS DURING EARLY MAMMALIAN EMBRYOGENESIS[1]

[1] Parts of the research in this chapter has been published as Shibata et al., 2011
Data presented in this chapter is primarily the contribution of Kristin E. Murphy (Blauvelt) to the publication above. Relevant data contributed by other authors are included in some of the figures. These contributions are acknowledged in the figure legends.

ABSTRACT

TRIM28 is a transcriptional regulator that is essential for a broad range of biological processes, including post-implantation embryonic development (Cammas et al., 2000; Iyengar and Farnham, 2011). TRIM28 functions as a transcriptional co-repressor by recruiting chromatin modifying factors, and its target specificity is believed to reside in its ability to bind different KRAB domain Zinc Finger proteins. Although KRAB Zinc finger proteins represent the largest family of transcriptional regulators in mammals, the functions of individual members of this family are largely unknown (Urrutia, 2003). Previous work from our laboratory provided genetic evidence that TRIM28 functions with the KRAB Zinc Finger protein ZFP568 to control embryo morphogenesis (García-García et al., 2008; Shibata and García-García, 2011; Shibata et al., 2011). Here, I demonstrate at the molecular level that TRIM28 physically interacts with ZFP568, and is required for ZFP568 to mediate transcriptional repression. I characterized molecularly the *Trim28*$^{chatwo}$ allele and found that the *chatwo* mutations impair the repressive activity of TRIM28. In addition, the *chatwo* mutation affects the stability of TRIM28-ZFP568 protein complexes. In agreement with the phenotypic similarities between *Zfp568*$^{chato}$ and *Trim28*$^{chatwo}$ embryos, I provide evidence that ZFP568 requires TRIM28 to a greater extent than other KRAB Zinc Finger proteins, supporting a model in which KRAB Zinc Finger proteins differentially require TRIM28 during embryonic development.




severe in *chatwo* embryos than in *chato* embryos (Fig. 1H, I, bracket). In addition, *chatwo*

**Fig. 2.1. *chatwo* creates a hypomorphic allele of Trim28.** (A-C) Whole-mount in situ hybridizations on wild-type (A), *Trim28*$^{chatwo}$ (B), and *Trim28*$^{KO}$ (C) embryos dissected at E8.5 using a probe for brachyury. (D) Western blot using anti-TRIM28 antibody and lysates from wild type (lanes 1-5) and *chatwo* (lane 6) embryos. Lanes 1 to 5 represent lysates from approximately 4, 2, 1, 0.5, and 0.25 wild-type embryos, respectively. Anti-GAPDH antibody was used as a loading control (lower panel). The TRIM28 antibody was raised against part of the coiled-coil and HP1-binding domain and was still able to recognize the *chatwo* mutant protein. (E) qRT-PCR analysis of *Trim28* expression in wild-type and *Trim28*$^{chatwo}$ embryos. (F) Domain structure of TRIM28 showing the location of *chatwo* mutations (red lettering). Sequence alignments show the conservation of the CH residues mutated in *chatwo* (highlighted in yellow) in mouse, human, and Xenopus TRIM28, as well as in other mouse TIF1 family members. (G-I) Images of wild type (G), *Trim28*$^{chatwo}$ (H) *Zfp568*$^{chato}$ (I) embryos dissected at E8.5. Arrows point to yolk sac blisters and brackets highlight areas of the yolk sac not affected by blisters. All panels in Fig. 1 were contributed by Maho Shibata. Parts of this figure were modified from Shibata et al., 2011.

extaembryonic phenotypes include defects not observed in *chato* embryos, suggesting that the *chatwo* mutations affect some morphogenetic roles of TRIM28 that are not shared by ZFP568. (Shibata and García-García, 2011; Shibata et al., 2011).

Here, I show that TRIM28 physically interacts with ZFP568 and is required to mediate its transcriptional repression, providing further evidence that the two proteins function in a shared molecular process. I determined that the *chatwo* mutations in TRIM28 reduce repressive activity of TRIM28, and affect the ability of TRIM28 to mediate ZFP568 repressive activity, suggesting that this molecular defect contributes to the morphogenetic defects in *chatwo* mutant embryos. Furthermore, I found that the hypomorphic *chatwo* allele of TRIM28 encodes a protein that is less stable than wild-type TRIM28 protein. Data presented here also indicates that ZFP568 repressive activity has a greater sensitivity to TRIM28 levels than other KRAB Zinc Fingers. Therefore, my results provide strong molecular evidence for a differential requirement for TRIM28 by KRAB Zinc Finger proteins during embryonic development.

## MATERIALS AND METHODS

### Yeast two-hybrid assays

GAL4 DNA-binding domain (DBD) and activation domain (AD) plasmids were sequentially transformed into AH109 yeast strain using Matchmaker GAL4 Two-Hybrid System 3 (Clontech). Colonies were re-plated onto Ade-His Leu-Trp- or Leu-Trp- X-alpha-gal plates.

### Cell culture

HEK293 or HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen). For immunoprecipitation, cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1% Triton X-100, 0.05% SDS and protease inhibitors. Immunoprecipitations were performed using 2-3 µl of antibody and 25 µl protein A/G agarose beads (Santa Cruz Biotechnology). For luciferase assays, HEK293T cells were transfected with pGL35XUAS firefly luciferase reporter, a GAL4DBD effector plasmid (GAL4DBD-ZFP568, GAL4DBD-TRIM28, GAL4DBD-TRIM28chatwo, GAL4DBD-TRIM286KR, GAL4DBD-ZFP57, or GAL4DBD-ZFP809), and control pRL *Renilla* luciferase plasmids. Total amount of DNA transfected was held constant by co-transfecting pCMV-MYC vector as needed. Cells were assayed with the Dual-Luciferase Reporter System (Promega) 24 hours after transfection. For small interfering RNA (siRNA) knockdown, 8 pmol of *Trim28* siRNAs #1 (19779), #2 (19778) or non-silencing siRNA (Ambion) was transfected using Lipofectamine RNAiMax (Invitrogen). Cells were transfected with GAL4DBD effector plasmids, luciferase reporter plasmids, and control *Renilla* luciferase plasmids 24 hours after siRNA transfection and luciferase was assayed after another 48 hours. For each luciferase assay, duplicate transfections and replicate lysates were measured for each condition ($n$=4). Firefly luciferase expression was normalized to *Renilla* to control for transfection efficiency. Percent luciferase expression was calculated compared with GAL4DBD. Lysates loaded for western blotting were normalized to *Renilla* expression. Statistical analysis was performed using paired, two-tailed $t$-test.

**Reticulocyte translation assays**

Translation was assayed using the TNT Coupled Reticulocyte Lysate and Transcend Non-Radioactive Translation Detection Systems (Promega) in the presence of 1 μg plasmid DNA and 1 μl of transcend tRNA (biotinylated lysine). Translated protein was visualized by Western blotting using Streptavidin-HRP (1:10,000).

**Antibodies**

The following antibodies were used for Western blotting, co-IP and/or immunofluorescence: anti-TRIM28 (H-300, Santa Cruz Biotechnology; 1:500), anti-GAL4DBD (RK5C1, Santa Cruz Biotechnology; 1:500-1:800), anti-Myc (9e10, Hybridoma Bank; 1:250-1:1000), anti-Flag (M2, Sigma-Aldrich; 1:500-1:700), anti-HA (11, Covance 1:250), anti-HA (Y11, Santa Cruz Biotechnology; 1:500), anti-GAPDH (AB9482, Abcam; 1:8000), anti-mouse/rabbit HRP (Jackson ImmunoResearch; 1:10,000).

**Constructs and primers**

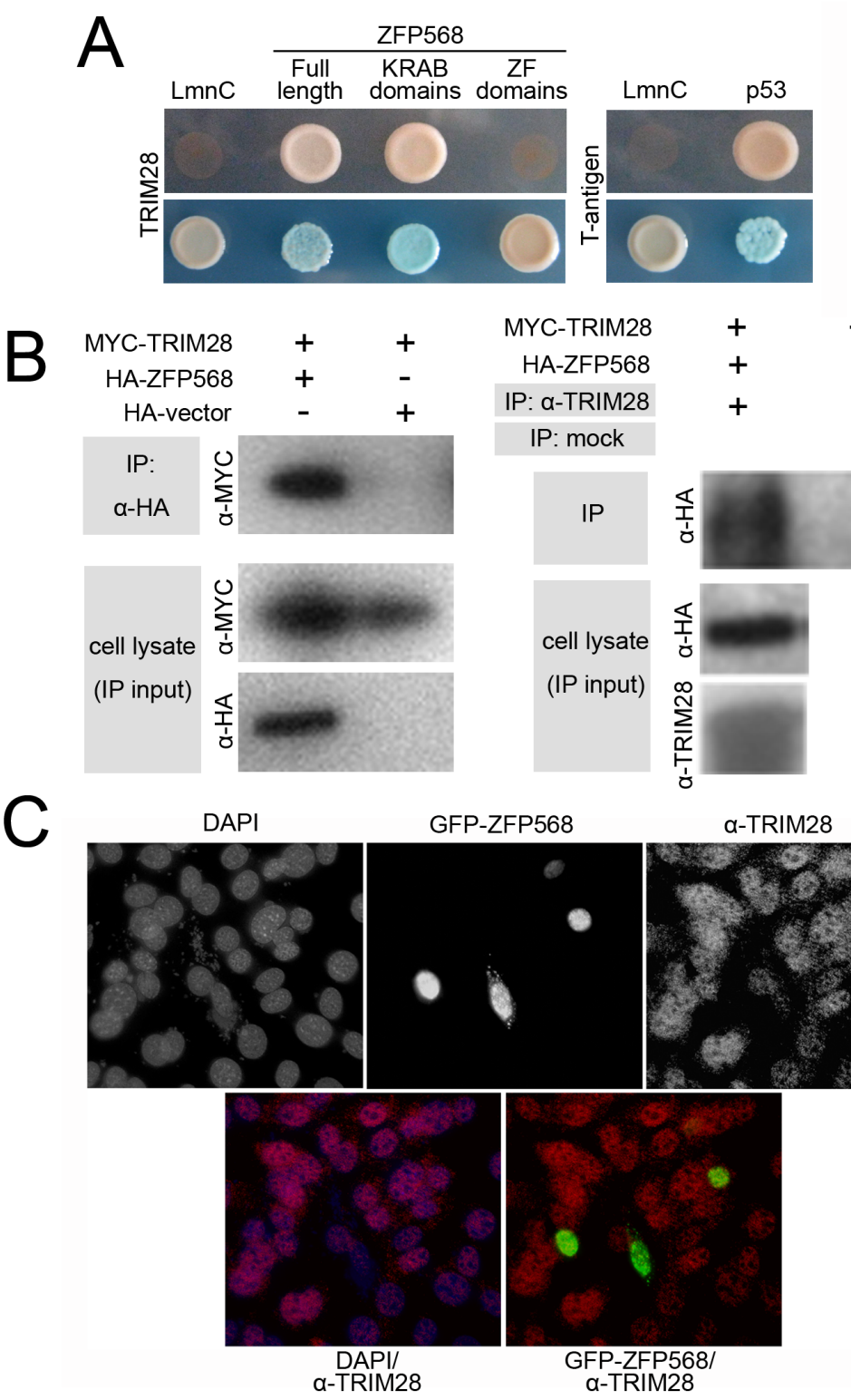
Plasmids pCDNA3.1-GAL4DBD-TRIM28, pCDNA3.1-GAL4DBD-TRIM286KR, pGL35XUAS firefly luciferase and pRL *Renilla* luciferase are described in Mascle et al. (Mascle et al., 2007). Yeast two-hybrid control constructs were obtained from Clontech. Other constructs were generated using primers as indicated below:

| construct | cloning | primers |
|---|---|---|
| pGADT7-TRIM28 | EcoRI digest from PCR-amplified cDNA fragment | TTAGAATTCTTGCGTGATAGTGGCAGTAAGG, TAAGAATTCTGGTTCTACCAGCACAGCAG |
| pGBKT7-DBD-ZFP568 (full length) | EcoRI digest from PCR-amplified cDNA fragment | AATTGTCGACACCCAGCCTTGAAATACCAG, AATTGTCGACTGTTATCCACCACAGGGTTTT |

| | | |
|---|---|---|
| pGBKT7-DBD-ZFP568 (KRAB domains) | EcoRI digest from PCR-amplified cDNA fragment | ATTGTCGACACCCAGCCTTGAAATACCAG, AATTGTCGACCTCACTGGCCTTTGCCTTAC |
| pGBKT7-DBD-ZFP568 (ZF domains) | EcoRI digest from PCR-amplified cDNA fragment | AATTGTCGACGTAAGGCAAAGGCCAGTGAG, AATTGTCGACTGTTATCCACCACAGGGTTTT |
| pAcGFP-ZFP568 | BamHI-XhoI digest from PCR-amplified cDNA fragment | TCAGATCTCGAGATGGAGCGCTTGTCCCAGATG, ACCGGTGGATCCCGTTCACTCCTCCGTCCTGTATG |
| pCMV-Myc-TRIM28 | EcoRI digest from PCR-amplified cDNA fragment | TTAGAATTCGTCCGGCTGCTTCCTCAG, TAAGAATTCGTGGTTCTACCAGCACAGCAG |
| chatwo site directed mutagenesis | SD mutagenesis on pGADT7-TRIM28, pCDNA3-Flag-TRIM28, pcDNA3.1-GAL4DBD-TRIM28, pCMV-Myc-TRIM28 | CCTGGCCCTGTTCTGGAATGAACCATGCCGTC, GACGGCATGGTTCATTCCAGAACAGGGGGAGG |
| Trim28 for qRT-PCR | | GTGGAGCCTCATGGTGAGAT, TACTTCCATGGGCTGGCTAC |
| Gapdh for qRT-PCR | | ACTGCCACCCAGAAGACTGT, GATGCAGGGATGATGTTCTG |
| pGBKT7-ZFP57 WT | EcoRI digest from PCR-amplified cDNA fragment | TTAGAATTCTTAGGACCAGCCTGGCATTAC, TAAGAATTCGAGCATGGGTGGTGTGAAG |
| pGBKT7-ZFP809 WT | EcoRI digest from PCR-amplified cDNA fragment | TTAGAATTCTCGGAGACCGAGTCACAGG, TAAGAATTCGCAGAGAACACACTGGGGATA |
| GAL4DBD-ZFP568 WT | EcoRI digest from pGBKT7-ZFP568 | |
| GAL4DBD-ZFP57 WT | EcoRI digest from PCR-amplified cDNA fragment | TTAGAATTCTAGGACCAGCCTGGCATTAC, TAAGAATTCGAGCATGGGTGGTGTGAAG |
| GAL4DBD-ZFP809 WT | EcoRI digest from PCR-amplified cDNA fragment | TTAGAATTCCGGAGACCGAGTCACAGG, TAAGAATTCGCAGAGAACACACTGGGGATA |
| HA-ZFP568 | SfiI-NotI digest from pGBKT7-ZFP568 | |
| Flag-TRIM28 | EcoRI-XbaI digest from GAL4DBD-TRIM28 into pcDNA3.1-Flag vector | |
| Flag-TRIM28-6KR | EcoRI digest from GAL4DBD-TRIM286KR | |

## RESULTS

### TRIM28 interacts with ZFP568




protein was targeted to the promoter of a luciferase gene via UAS sites (Fig. 2.3A). GAL4DBD-

**Fig. 2.2. ZFP568 interacts with TRIM28.** (A) Yeast two-hybrid assays showing interaction of GAL4DBD-ZFP568 (full length and KRAB domains constructs) with GAL4AD-TRIM28, as indicated by growth on Ade-, His-, Leu-, Trp- media (top panel) and blue colony color in Leu-, Trp-, X-alpha-gal plates (lower panel). A construct containing only ZFP568 ZF domains did not show interaction with TRIM28 in this assay. p53 interaction with SV40 large T-antigen was used as a positive control and Lamin C (LmnC) interaction with SV40 large T-antigen was used as a negative control. (B) HA-ZFP568 and MYC-TRIM28 co-immunoprecipitate (co-IP) when transfected in HEK293 cells when anti-HA antibody was used to immunoprecipitate. HA-ZFP568 and MYC-TRIM28 also co-IP when anti-TRIM28 antibody was used to immunoprecipitate (C) Immunohistochemistry with anti-TRIM28 antibodies showing the sub-cellular localization of endogenous TRIM28 (red) and transfected GFP-ZFP568 (green) in NIH3T3 cells. Samples were co-stained with DAPI. Parts of this figure were modified from Shibata et al., 2011.

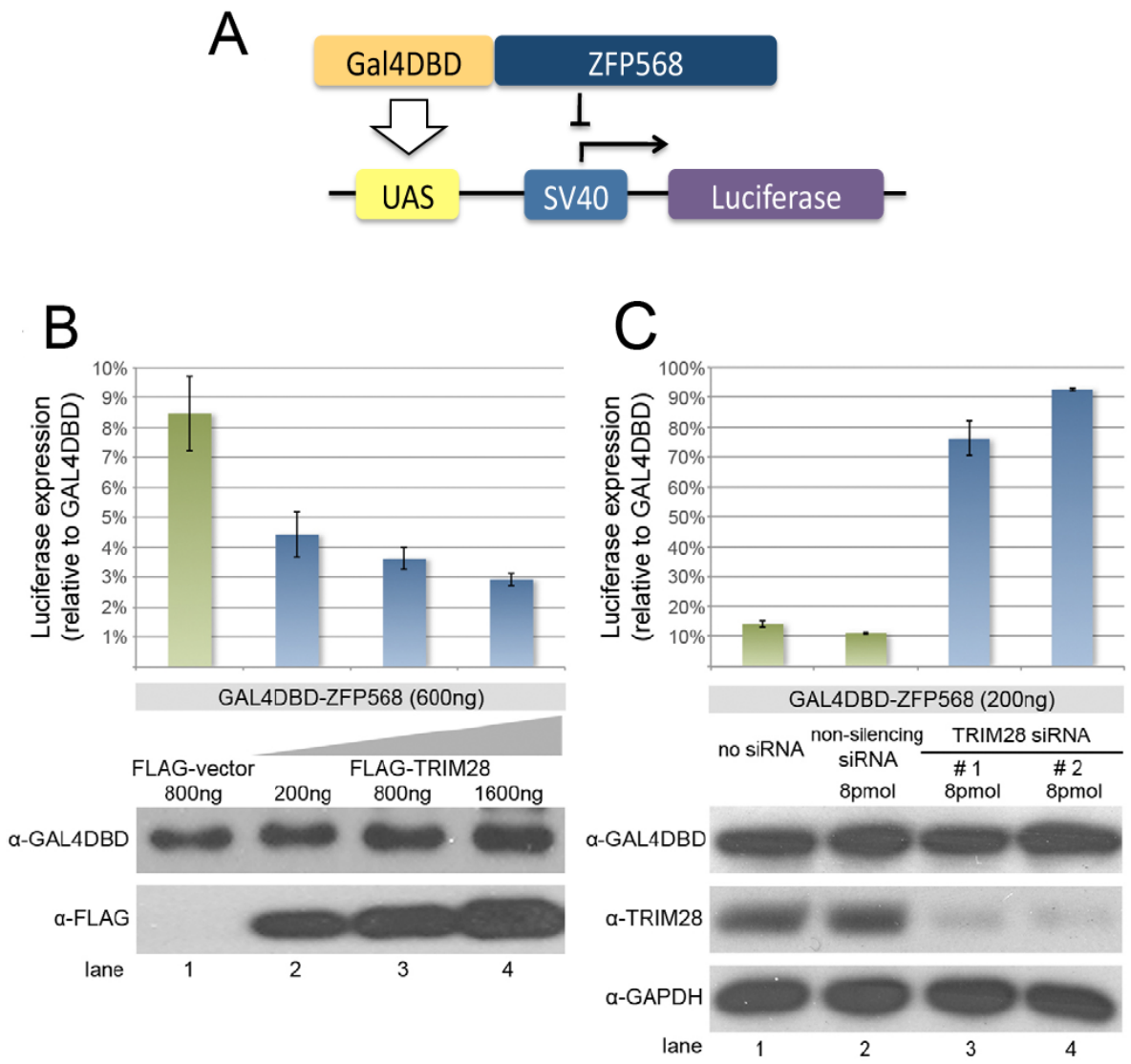


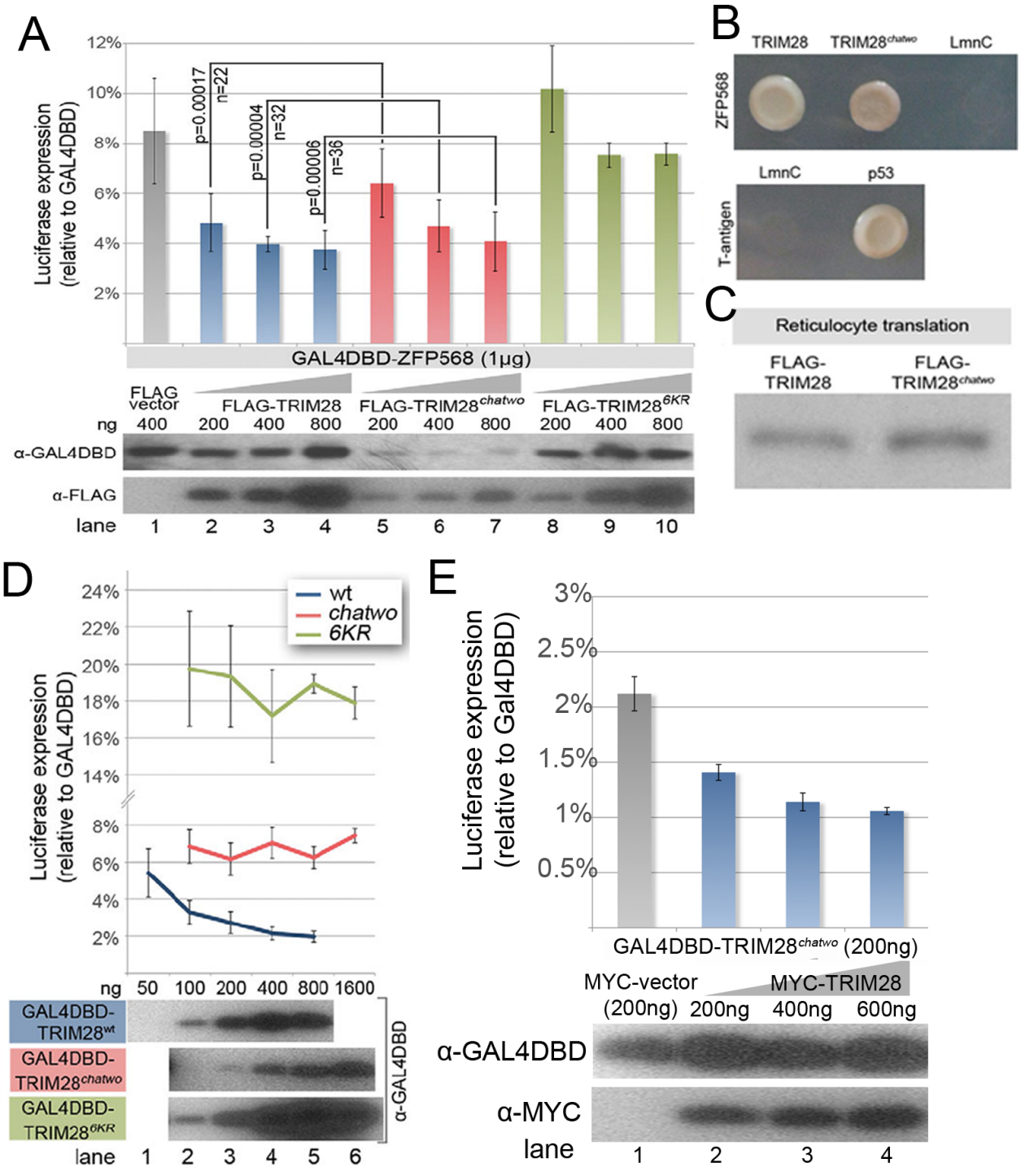

**Fig. 2.3**. **TRIM28 mediates ZFP568 repressive activity**. (A) Cartoon depicting experimental design shows a chimeric GAL4DBD-ZFP568 protein recruited to upstream activating sequences (UAS). ZFP568 repressive activity results in decreased Luciferase gene expression. (B) Quantification of luciferase expression in HEK293T cells, in the presence of GAL4DBD-ZFP568 and absence (green)/presence (blue) of increasing amounts of FLAG-TRIM28. (C) Quantification of luciferase expression in HEK293T cells, in the presense of GAL4DBD-ZFP568 with (blue) and without (green) treatment with *Trim28* siRNA pool, normalized to GAL4DBD-ZFP568 repressive activity and set to 100% (GAL4DBD empty vector (100%). Error bars represent s.d. Western blots show levels of ZFP568-Gal4DBD and FLAG-TRIM28 proteins normalized for transfection efficiency. GAPDH serves as loading control for the siRNAs, which were introduced through an independent transfection event (see Materials and methods). Results in B represent one of six experiments showing similar results. Parts of this figure were modified from Shibata et al., 2011.

Since *chatwo* mutants resemble *chato* embryos, I investigated the effects of the *chatwo* mutations on TRIM28 mediated ZFP568 repression. The *6KR* mutant allele of TRIM28 prevents SUMOylation at 6 different acceptor sites and has been previously shown to impair repressive activity of TRIM28 (Mascle et al., 2007). In the presence of increasing levels of FLAG-TRIM28$^{6KR}$, Luciferase expression from GAL4DBD-ZFP568 repressive activity was not significantly relieved (Fig. 2.4A, green bars compared with grey bar), suggesting that TRIM28$^{6KR}$ cannot mediate ZFP568 repressive activity. In contrast, overexpressed FLAG-TRIM28$^{chatwo}$ did significantly enhance ZFP568 transcriptional repression in a dose-dependent manner (Fig. 2.4A, red bars

compared with grey bar), implying that it can form a complex with ZFP568 to mediate its repressive activity. In support of this, TRIM28$^{chatwo}$ maintained the ability to interact with ZFP568 by yeast two-hybrid (Fig. 2.2B). In the luciferase assay, ZFP568 repressive activity was, however, consistently lower in response to overexpressed FLAG-TRIM28$^{chatwo}$ compared with FLAG-TRIM28 (Fig. 2.4A, red bars compared with blue bars). These data indicate that the hypomorphic *chatwo* mutations reduce, but do not completely abolish, the ability of TRIM28 to mediate ZFP568 repression.

In addition to its effects on ZFP568-mediated repression, overexpressed FLAG-TRIM28$^{chatwo}$ also affected protein stability of ZFP568-TRIM28 complexes (Fig. 2.4A). Upon similar transfection conditions, I observe that FLAG-TRIM28$^{chatwo}$ protein levels were lower than FLAG-TRIM28 levels, and that GALDBD-ZFP568 protein levels were also reduced when FLAG-TRIM28$^{chatwo}$ was overexpressed (Fig. 2.4A, western blot lanes 5-7 comapared to lanes 2-4). These *in vitro* data are in agreement with a previously observed reduction in TRIM28 protein levels in *chatwo* embryos *in vivo* (Fig. 1E). Additionally, several other reports have shown that KRAB domain stability is dependent on TRIM28 (Peng and Wysocka, 2008; Wolf and Goff, 2009). This effect on protein stability was specific to the *chatwo* mutations, as FLAG-TRIM28$^{6KR}$ overexpression did not cause reduced ZFP568-TRIM28 protein levels (Fig. 2.4A, western blot lanes 8-10). In order to determine whether the *chatwo* mutations decrease TRIM28 protein levels by disrupting translation efficiency, we utilized an *in vitro* reticulocyte translation assay. Both FLAG-TRIM28 and FLAG-TRIM28$^{chatwo}$ were efficiently translated *in vitro*, and similar protein levels were detected by Western blot (Fig. 2.4C). This result suggests that the *chatwo* mutations do not affect the transcription efficiency or translation efficiency of TRIM28,

but rather they affect TRIM28 protein stability and/or rate of protein degradation post-




amounts of GAL4DBD-TRIM28 and GAL4DBD-TRIM28$^{chatwo}$ proteins were similar, the repressive activity of GAL4DBD-TRIM28$^{chatwo}$ was still significantly lower than that of

**Fig. 2.4**. ***chatwo* mutations disrupt TRIM28 stability and repressive activity.** (A) Luciferase expression from a 5xUAS-luciferase reporter in HEK293T cells in the presence of GAL4DBD-ZFP568 and either FLAG empty vector (gray bar), increasing amounts of FLAG-TRIM28 (blue), Flag-TRIM28$^{chatwo}$ (red) or Flag-TRIM28$^{6KR}$ (green). (B) Yeast two-hybrid assays showing interaction of GAL4DBD-ZFP568 with GAL4AD-TRIM28$^{chatwo}$, as indicated by growth on Ade-, His-, Leu-, Trp- media. p53 interaction with SV40 large T-antigen was used as a positive control and Lamin C (LmnC) interaction with SV40 large T-antigen was used as a negative control. (C) Western blot using anti-Streptavadin-HRP antibody following *in vitro* rabbit reticulocyte translation of FLAG-TRIM28 and FLAG-TRIM28$^{chatwo}$ plasmids. (D) Luciferase expression from a 5xUAS-luciferase reporter in HEK293T cells in the presence of increasing amounts of GAL4DBD-TRIM28$^{wt}$ (blue line), GAL4DBD-TRIM28$^{chatwo}$ (red line) or GAL4DBD-TRIM28$^{6KR}$ (green line). (E) Luciferase expression from a 5xUAS-luciferase reporter in HEK293T cells in the presence of GAL4DBD-TRIM28$^{chatwo}$ and either empty vector (gray bar) or increasing amounts of MYC-TRIM28 (blue bars). In luciferase experiments, luciferase expression is plotted as the percentage relative to GAL4DBD empty vector (100%). Error bars represent s.d. Results in A represent one of nine experiments showing similar results. *P*-values were calculated using data from all nine experiments (*n*=total number of data points). Levels of GAL4DBD-ZFP568 in the presence of FLAG-TRIM28$^{chatwo}$ were significantly lower compared with FLAG-vector conditions in six out of of seven western blot experiments. Western blots show levels of chimeric proteins normalized for transfection efficiency. Parts of this figure were modified from Shibata et al., 2011.

GAL4DBD-TRIM28 (Fig. 2.4D, compare 200ng GAL4DBD-TRIM28 with 800ng GAL4DBD-TRIM28$^{chatwo}$). Altogether, these results indicate that the *chatwo* mutations reduce TRIM28 repressive activity independently of affecting TRIM28 protein stability.

In the luciferase reporter experiments presented here, endogenous TRIM28 is present within HEK293T cells. Because TRIM28 is able to form homo-oligomers ((Peng et al., 2002a), I explored whether endogenous wild type TRIM28 protein expressed by HEK293T cells influences TRIM28$^{chatwo}$ repressive activity in luciferase reporter assays. The repressive activity of GAL4DBD-TRIM28$^{chatwo}$ was significantly enhanced in a dose-dependent manner when increasing levels of MYC-TRIM28 were co-transfected (Fig. 2.4E). Because MYC-TRIM28 cannot be directly recruited to the luciferase promoter, this result suggests that wild type TRIM28 enhances luciferase repression by oligomerizing with GAL4DBD-TRIM28$^{chatwo}$. This oligomerization could enhance repressive activity in two possible ways: wild type TRIM28 could contribute to recruit repressive co-factors more efficiently and/or to enhance the stability of TRIM28/GAL4DBD-TRIM28$^{chatwo}$ complexes. Either way, these results indicate that TRIM28$^{chatwo}$ repressive activity in luciferase reporter assays is likely higher than that of TRIM28 in *chatwo* mutant embryos, where wild type protein is only present at early embryonic stages (maternal contribution). Nevertheless, my results from luciferase experiments indicate that the *chatwo* mutations disrupt the repressive activity and stability of TRIM28.

**TRIM28 is differentially required for KRAB Zinc Finger repressive activity**

TRIM28 is proposed to be a universal co-repressor for all KRAB Zinc Finger proteins (Urrutia, Moosman 1996, Kim 1996, Abrink 2001). Yet, hypomorphic *Trim28*$^{chatwo}$ embryos

distinctly resemble *Zfp568*$^{chato}$ embryos. Therefore, I hypothesized that ZFP568 requires TRIM28 to a greater extent than other KRAB Zinc Finger proteins. To test this, I reduced

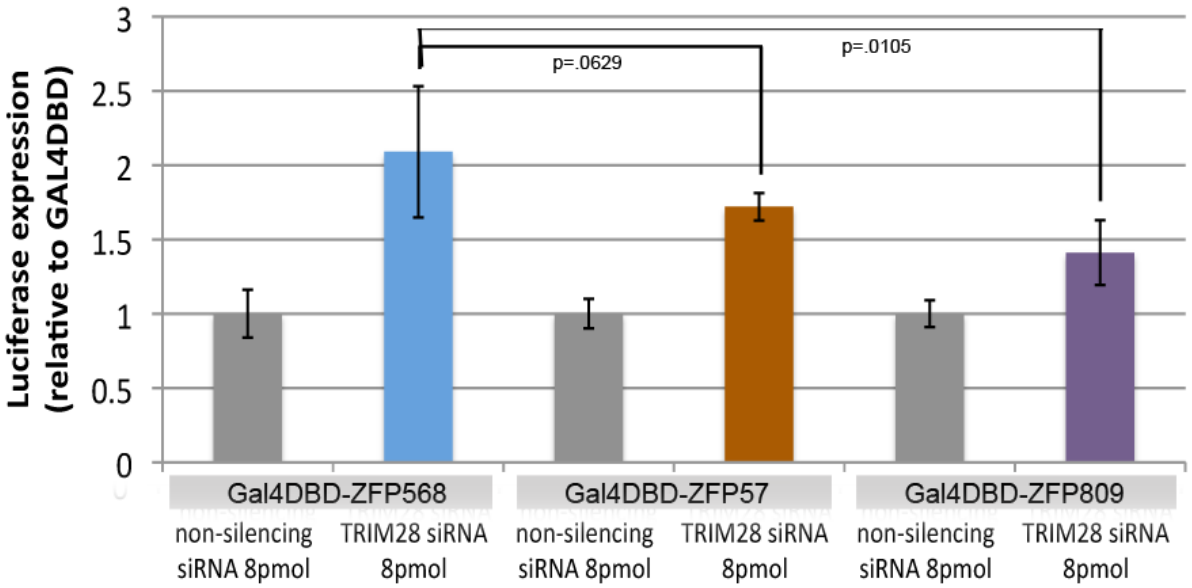



**Fig. 2.5. TRIM28 is required by ZFP568 more than other KRAB Zinc Finger proteins.**
Quantification of luciferase expression from a 5xUAS-luciferase reporter in HEK293T cells in the presence of GAL4DBD-ZFP568, GAL4DBD-57, or GAL4DBD-ZFP809 with treatment with non-silencing siRNA(gray bars) or *Trim28* siRNA #2 (colored bars). Luciferase expression is plotted as the percentage relative to GAL4DBD empty vector, and normalized to non-silencing siRNA expressions (1). Error bars represent s.d.



**TRIM28 functions in a repressive complex with ZFP568 to control morphogenesis**

The morphogenetic defects between *chato* and *chatwo* embryos previously suggested that TRIM28 and ZFP568 function in a common molecular process (Shibata et al., 2011). Here, I confirmed that TRIM28 physically interacts with ZFP568 and co-localizes with ZFP568 in the nucleus of mammalian cells. I showed that ZFP568 functions as a transcriptional repressor in luciferase reporter assays, and that TRIM28 levels mediate ZFP568 repressive activity. In addition, my results indicated that ZFP568 requires TRIM28 for repressive activity. Therefore, in

addition to ZFP568$^{chato}$ and TRIM28$^{chatwo}$ interacting genetically during embryogenesis, TRIM28 and ZFP568 interact molecularly to achieve transcriptional repression. Based on these results, I infer that TRIM28 and ZFP568 function together in a repressive complex to control mammalian embryonic morphogenesis.

### *chatwo* mutations disrupt repressive activity and protein stability functions of TRIM28

The *chatwo* mutations within the bromodomain of TRIM28 create a hypomorphic allele (Shibata et al., 2011). Interestingly, I determined that the ability of TRIM28$^{chatwo}$ to mediate ZFP568 repressive activity is reduced compared to wild type TRIM28. In addition, I showed that the *chatwo* mutations generally affect TRIM28 repressive activity when directly recruited to a gene promoter. I found that the *chatwo* mutations reduce the protein stability and/or rate of degradation of both TRIM28 and ZFP568 proteins, indicating that the TRIM28-ZF568 complex is destabilized by the *chatwo* mutations. However, decreased protein levels alone do not account for the effect the *chatwo* mutations have on TRIM28 repressive activity. Therefore, the mutations compromise TRIM28 functions through two different molecular mechanisms; protein instability and reduced repressive activity. These defects provide a molecular explanation for the embryonic lethality of *chatwo* mutants.

The *chatwo* mutations, located within the bromodomain of TRIM28, map to a predicted interface between the PHD and bromodomains, which cooperatively binds to chromatin modifiers such as SETDB1 and CHD3 (Schultz et al., 2001, 2002). I favor a hypothesis in which the *chatwo* mutations disrupt the folding of TRIM28, affecting its ability to form fully functional transcriptional repressor complexes and the stability of TRIM28-KRAB Zinc Finger protein

complexes. In the context of the embryo, I propose that TRIM28$^{chatwo}$ retains a repressive activity that is sufficient to accommodate early functions of TRIM28, allowing *chatwo* embryos to bypass the early lethality of *Trim28* knockout embryos.

**KRAB Zinc Finger proteins differentially require TRIM28 during embryogenesis**

Due to the strikingly similar morphogenetic defects between *chato* and *chatwo* embryos, I explored whether the *chatwo* mutations specifically affect ZFP568-dependent functions of TRIM28. TRIM28 is thought to be a universal co-repressor for all KRAB Zinc Finger proteins, and I presented molecular evidence that TRIM28 is required, not only for ZFP568-mediated repression, but also for repressive activity of other KRAB domain proteins. I also showed that ZFP568 has a greater requirement for TRIM28 protein levels for its repressive activity than the other KRAB Zinc Finger proteins. This result presents the possibility that the *chatwo* mutations impair ZFP568 functions more severely than the functions of other KRAB Zinc Finger proteins, either through protein instability or mechanisms that affect their repressive activities. Nonetheless, this provides an explanation for the similar developmental arrest and morphogenetic defects between *chato* and *chatwo* embryos. I speculate that TRIM28$^{chatwo}$ can function sufficiently with co-factors during early stages of embryogenesis, but cannot fulfill the functional requirements of TRIM28-ZFP568 repressive complexes at later stages.

ACKNOWLEDGEMENTS

I thank Drs. Ken Kemphues, John Schimenti, Mariana Wolfner, and members of the laboratory for helpful discussions. We also thank Drs. Muriel Aubry, Florence Cammas, Xavier Mascle, and Ling Qi, for mice, equipment, and reagents.

# CHAPTER 3

# THE TRANSCRIPTIONAL REPRESSION ACTIVITY OF KRAB DOMAIN ZINC FINGER PROTEINS DOES NOT CORRELATE WITH THEIR ABILITY TO RECRUIT THE TRANSCRIPTIONAL CO-REPRESSOR TRIM28[2]

[2] Data presented in this chapter is primarily the contribution of Kristin E. Murphy. Relevant data contributed by other members of the laboratory are included in some figures. These contributions are acknowledged in the figure legends.

ABSTRACT

KRAB domain Zinc Finger proteins represent the largest family of transcriptional regulators in higher vertebrates (Urrutia, 2003). Although the functions of individual proteins within this family are largely unknown, all of them are proposed to function as transcriptional repressors. This assumption is based on the fact that the majority of KRAB motifs examined to date represses transcription in reporter assays and binds the co-repressor TRIM28 (Urrutia, 2003). By studying mutations in KRAB Zinc Finger protein ZFP568, I found that some KRAB motif amino acid residues are more critical than others to mediate transcriptional repression. Significantly, my results also revealed that TRIM28 binding is not sufficient for KRAB domain repressive activity. By analyzing the repressive activity and ability to bind TRIM28 of a wider collection of KRAB domain proteins, I showed that repressive activities of KRAB domain proteins vary broadly amongst different family members, and that the repressive activities of KRAB Zinc Finger proteins does not necessarily correlate with their ability to bind TRIM28. Overall, this study provides novel contributions to the current understanding of the transcriptional roles of KRAB Zinc Finger proteins.

## INTRODUCTION

KRAB Zinc Finger proteins consist of one or more conserved KRAB domains at their amino terminal end, and a variable number of C2H2 Zinc Finger domains at their carboxyl terminal end (Margolin et al., 1994; Urrutia, 2003). KRAB domain protein encoding genes are present only in tetrapod vertebrates, yet there are over 400 KRAB domain proteins encoded in the human and mouse genomes (Urrutia, 2003; Sripathy et al., 2006; Iyengar and Farnham, 2011). Individual KRAB Zinc Finger proteins have been shown to regulate important biological processes such as ES cell retroviral silencing, imprinting, embryonic morphogenesis, mammary gland development, and sex-specific pubertal timing (Li et al., 2008; Wolf and Goff, 2009; Krebs et al., 2012; Oliver et al., 2012). Therefore, a thorough understanding of how these proteins function at a molecular level is crucial.

The repressive activities of KRAB domains have been shown to depend on their interaction with TRIM28 (Friedman et al., 1996; Sripathy et al., 2006), and the recruitment of chromatin modifier proteins that repress transcription (Sripathy et al., 2006). KRAB domain mutations that disrupt the interaction with TRIM28 correlate with a loss of KRAB repressive activity (Margolin et al., 1994; Friedman et al., 1996). However, it is not known whether TRIM28 is sufficient for KRAB-mediated transcriptional repression and whether all KRAB Zinc Finger proteins require TRIM28. This study provides a comprehensive analysis of TRIM28 requirements by ZFP568, as well as other KRAB Zinc Finger proteins.

ZFP568 is a member of the KRAB domain protein family that is required for mouse embryonic development (García-García et al., 2008; Shibata and García-García, 2011). *chato*, an ENU induced mutation in mouse *Zfp568*, results in embryonic lethality and severe morphogenetic defects. The *chato* mutation introduces a non-conservative amino acid change in the first of two canonical KRAB motifs in ZFP568 and completely disrupts ZFP568 function (García-García et al., 2008). Here, I investigated the requirements of ZFP568 activity for TRIM28. I found that TRIM28 binding is not sufficient to mediate ZFP568 repressive activity. I also determined that specific amino acid residues within the KRAB motifs of ZFP568 influence its repressive activity. I expanded the investigation to mouse and human KRAB domain proteins in addition to ZFP568, and discovered that some proteins contain non-repressive KRAB domains. I found that a subset of KRAB Zinc Finger proteins lack efficient transcriptional repressive activity, despite binding TRIM28. Conversely, other KRAB domain proteins can repress transcription in the absence of TRIM28 binding. These analyses challenge the assumptions that repressive activity of the KRAB motif exclusively depends on TRIM28, and that all KRAB Zinc Finger proteins function as transcriptional repressors (Urrutia 2003).

## MATERIALS AND METHODS

### Luciferase Assays

HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen) with pGL3-5XUAS Firefly luciferase reporter, a GAL4DBD effector and pRL Renilla luciferase plasmids. Total amount of DNA transfected was held constant by co-transfecting with pCMV-MYC as needed.

Cells were assayed with the Dual-Luciferase Reporter System (Promega) 24 hours after transfection. For small interfering RNA (siRNA) knockdown, 8 pmol of Trim28 siRNAs #1 (19779), #2 (19778) or non-silencing siRNA (Ambion) was transfected using Lipofectamine RNAiMax (Invitrogen). Cells were transfected with luciferase effectors and reporters 24 hours after siRNA transfection and luciferase was assayed after another 48 hours. For each luciferase assay, duplicate transfections and replicate lysates were measured for each condition (n=4). Luminescence was read using a luminometer plate reader (BioTek) Firefly luciferase luminescence was normalized to Renilla luciferase luminescence to control for transfection efficiency. Fold repression was calculated compared to GAL4DBD. Lysates loaded for western blotting were normalized to Renilla expression. Statistical analysis was performed using paired, two-tailed t-test.

**Immunoprecipitations and Western Blots**

HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen). For immunoprecipitation, cells were lysed in buffer containing 250 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1% Triton X-100, 0.05% SDS and protease inhibitors. Immunoprecipitations were performed using 2 µl of antibody and 50 µl Protein G Dynabeads (Invitrogen). Western blots were performed using standard protocols and analyzed using the Odyssey Digital Imager (LiCor).

**Yeast two-hybrid assays**

GAL4DBD and AD fusion plasmids were sequentially transformed into AH109 yeast strain using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech). Colonies were re-plated onto Ade-, His-, Leu-, Trp- or Leu-, Trp- X-alpha-gal plates. β-galactosidase activity was measured using the pellet X-gal (PXG) assay (Möckli and Auerbach, 2004). Cyan Color development was quantified using ImageJ.

**Computational analysis**

A complete list of KRAB domains and amino acid sequences in the mouse genome was obtained from Pfam. A non-redundant list was created using Find Duplicate Sequences Program (Saurin et al., 1996). Motif Logo was created using WebLogo3 (http://weblogo.threeplusone.com/create.cgi).

**Antibodies**

The following antibodies were used for western blotting and/or co-immunoprecipitation (co-IP), anti-TRIM28 (H-300, Santa Cruz Biotechnology; 1:500), anti-GAL4DBD (RK5C1, Santa Cruz Biotechnology; 1:500-1:800), anti-Flag (M2, Sigma-Aldrich; 1:500-1:700), mouse/rabbit IgG (Santa Cruz Biotechnology), anti-mouse/rabbit IR secondary dyes 800CW or 680LT (LiCor; 1:15,000).

**Constructs and primers**

Plasmids pCDNA3.1-GAL4DBD, pGL35XUAS firefly luciferase and pRL *Renilla* luciferase are described in Mascle et al., 2007 (Mascle et al., 2007). Yeast two-hybrid control constructs were obtained from Clontech. Other constructs were generated as described below:

| construct | cloning | primers |
|---|---|---|
| GAL4DBD-ZFP568 WT | EcoRI fragment from pGBKT7-ZFP568 | TTAGGTACCTCCTTAAAGGCAAAACTCTTCA, TTAGGTACCAGAAGCCAAGGACAGTTCCA |
| GAL4DBD-ZFP568 KRAB1 L-P | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP568 WT | TTAGGTACCTCGCCAGGAAGAAGAAGACAG, TTAGGTACCTCCTTAAAGGCAAAACTCTTCA |
| GAL4DBD-ZFP568 KRAB2 L-P | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP568 WT | TTAGGTACCTTTTTAAGGACATGGCCAAGGA, TTAGGTACCTTTTGGCTTCTGTCCTAGACACC |
| GAL4DBD-ZFP568 KRAB1 L-P KRAB2 L-P | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP568 KRAB1 L-P | TTAGCGGCCGCTCAACCTCTGGAACCCAGTGT, TTAGCGGCCGCGTGGTGTCCTGAGCTGTCCT |
| GAL4DBD-ZFP568 KRAB1 DV-AA | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP568 WT | CCTGTACAGGGAGATCATGCCGGAGAACTACAGC AACATG, CATGTTGCTGTAGTTCTCCGGCATGATCTCCCTGTA CAGG |
| GAL4DBD-ZFP568 KRAB2 DV-AA | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP568 WT | GTATCATGATGTGATGCCAGAGACCTTGGGCAACT, AGTTGCCCAAGGTCTCTGGCATCACATCATGATAC |
| GAL4DBD-ZFP568 ΔKRAB2 | Deletion of KRAB2 using KpnI PCR fragment from pcDNA3.1-GAL4DBD-ZFP568 WT | TTAGCGGCCGCTGATCAGCAATAGCTGTGTGAC, TTAGCGGCCGCGGGTAAGTTCTGAGCCACGA |
| GAL4DBD-ZFP568 ΔKRAB1 | Deletion of KRAB1 using KpnI PCR fragment from pcDNA3.1-GAL4DBD-ZFP568 WT | GTATCGAGATGTGATGCCGGAGAACTACAGCAACC , GGTTGCTGTAGTTCTCCGGCATCACATCTCGATAC |
| GAL4DBD-ZFP568 KRAB1 KRAB1 | KpnI restriction PCR fragment from pcDNA3.1-GAL4DBD-ZFP568 WT into pcDNA3.1-GAL4DBD-ZFP568 ΔKRAB2 | GGATTTGTACCGAGATGTAATGCCGGAAAATTACG GCAACCTG, CAGGTTGCCGTAATTTTCCGGCATTACATCTCGGTA CAAATCC |
| GAL4DBD-ZFP568 KRAB2 KRAB2 | KpnI restriction PCR fragment from pcDNA3.1-GAL4DBD-ZFP568 WT into pcDNA3.1-GAL4DBD-ZFP568 ΔKRAB1 | TTAGCGGCCGCAGGGGTGTGGGTCTGTGAG, TTAGCGGCCGCTGGGGTTTTAAACTGCTGGA |
| GAL4DBD-ZFP110 WT | NotI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TTAGCGGCCGCCGTGTTCGGGTCTTTCTAGG, TTAGCGGCCGCAGGCTTGAGGAAACAGCAAA |

| | | |
|---|---|---|
| GAL4DBD-ZFP110 KRAB1M-P | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP110 WT | CCTGTACAGGGAGATCATGCCGGAGAACTACAGCAACATG, CATGTTGCTGTAGTTCTCCGGCATGATCTCCCTGTACAGG |
| GAL4DBD-ZFP110 KRAB2L-P | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP110 WT | CCCTTTACCAGAAAGTGATGCCAGAAACCTTCAAGAACCTG, CAGGTTCTTGAAGGTTTCTGGCATCACTTTCTGGTAAAGGG |
| GAL4DBD-humanZNF568 WT | NotI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TTAGCGGCCGCTTTTGAGCCACATGATGGAAA, TTAGCGGCCGCGGGTAAGTTCTGAGCCACGA |
| GAL4DBD-humanZNF568 KRAB1LP | SD mutagenesis from pcDNA3.1-GAL4DBD-humanZNF568 WT | CTGTACAGGGATGTGATGCCGGAAACCTACAGCAGCCTGG, CCAGGCTGCTGTAGGTTTCCGGCATCACATCCCTGTACAG |
| GAL4DBD-humanZNF568 KRAB2LP | SD mutagenesis from pcDNA3.1-GAL4DBD-humanZNF568 WT | CAAAAACAGTGACATTCGAGGATGTGGCTGTGGAC, GTCCACAGCCACATCCTCGAATGTCACTGTTTTTG |
| GAL4DBD-humanZNF568 KRAB1LP KRAB2LP | SD mutagenesis from pcDNA3.1-GAL4DBD-humanZNF568 KRAB1LP | CAAAAACAGTGACATTCGAGGATGTGGCTGTGGAC, GTCCACAGCCACATCCTCGAATGTCACTGTTTTTG |
| GAL4DBD-humanZNF28 WT | NotI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TGTGGACCTCACACTGGAGGAATGGCAGC, GCTGCCATTCCTCCAGTGTGAGGTCCACA |
| GAL4DBD-humanZNF560 WT | NotI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | GCAGCAAATGAAGCCTGCTCACAGGGCTTTGTAC, GTACAAAGCCCTGTGAGCAGGCTTCATTTGCTGC |
| GAL4DBD-ZFP57 WT | EcoRI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | GGGCTTTGTACCGAGCTGTGATGCTGGAGAC, GTCTCCAGCATCACAGCTCGGTACAAAGCCC |
| GAL4DBD-ZFP57 KRABS-P | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP57 WT | CGAGATGTGATGCTGGACACCTACAGCAACC, GGTTGCTGTAGGTGTCCAGCATCACATCTCG |
| GAL4DBD-ZFP809 WT | EcoRI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TTAGAATTCTTGGACATTGCCGTGGACTTAT, TAAGAATTCTCAAGGTTACTCGAGTCCTGAA |
| GAL4DBD-ZFP809 KRABL-P | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP809 WT | TTAGCGGCCGCTTCTGTTCCGGAGCCTCTGTG, TAAGCGGCCGCTCTTTCCAGTGTCAGCTATGTCA |

| | | |
|---|---|---|
| GAL4DBD-KRAB1 KDV-EDV ΔKRAB2 | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP568 ΔKRAB2 | TTAGAATTCTTCCCTCTGTCACCTCCAAAAA, TAAGAATTCATGGGCACCTTTTGGTAGAA |
| GAL4DBD-KRAB1 QEEW-LEEW ΔKRAB2 | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP568 ΔKRAB2 | TTAGCGGCCGCTTCCATCAAATCCCATCATGC, TAAGCGGCCGCCCTGGGTTTTGGCACA |
| GAL4DBD-KRAB1 QRA-HRA ΔKRAB2 | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP568 ΔKRAB2 | TTAGCGGCCGCTAGGACGCTCCATGTCCTG, TAAGCGGCCGCAATCACAGGGTCCC |
| GAL4DBD-KRAB1 DVM-AVM ΔKRAB2 | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP568 ΔKRAB2 | TTAGAATTCTTCCTTGTTCCGTTCCTTTCAG, TAAGAATTCGTTCTTGGTTCTCCGCCATA |
| GAL4DBD-KRAB1 MLE-MLD ΔKRAB2 | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP568 ΔKRAB2 | TTAGCGGCCGCTACCTCCTGGAAGGAAGGGTA, TAAGCGGCCGCCGAGTCTTTTCAAGCACTATTTT |
| GAL4DBD-ZFP69 WT | EcoRI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TTAGAATTCTTGGACATTGCCGTGGACTTAT, TAAGAATTCTCAAGGTTACTCGAGTCCTGAA |
| GAL4DBD-ZFP617 WT | EcoRI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TTAGCGGCCGCTTCTGTTCCGGAGCCTCTGTG, TAAGCGGCCGCTCTTTCCAGTGTCAGCTATGTCA |
| GAL4DBD-ZFP446 WT | EcoRI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TTAGAATTCTTCCCTCTGTCACCTCCAAAAA, TAAGAATTCATGGGCACCTTTTGGTAGAA |
| GAL4DBD-ZFP496 WT | EcoRI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TTAGCGGCCGCTTCCATCAAATCCCATCATGC, TAAGCGGCCGCCCTGGGTTTTGGCACA |
| GAL4DBD-ZFP68 WT | EcoRI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TTAGCGGCCGCTAGGACGCTCCATGTCCTG, TAAGCGGCCGCAATCACAGGGTCCC |
| GAL4DBD-ZFP112 WT | EcoRI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TTAGAATTCTTCCTTGTTCCGTTCCTTTCAG, TAAGAATTCGTTCTTGGTTCTCCGCCATA |
| GAL4DBD-ZFP61 WT | EcoRI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TTAGCGGCCGCTACCTCCTGGAAGGAAGGGTA, TAAGCGGCCGCCGAGTCTTTTCAAGCACTATTTT |

| | | |
|---|---|---|
| GAL4DBD-ZFP819 WT | NotI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TTAGCGGCCGCACATGGCTGCTGACATGAAT, TAAGCGGCCGCTGAGCAAACAACTGCACA |
| GAL4DBD-ZFP13 WT | EcoRI restriction PCR fragment from cDNA clone into pcDNA3.1-GAL4DBD alone vector | TTAGAATTCTTGCTGTTTTTCCTGGCTCT, TAAGAATTCCCTGGGTGTTCTCACTGCTC |
| GAL4DBD-ZFP13 ΔKRAB | BamHI restriction PCR fragment from GAL4DBD-ZFP13 | TTAGGATCCTTGTCGCAACCCTAGAGGACAC, TTAGGATCCCCATCTTTCGCTCTCTGGTC |
| GAL4DBD-ZFP819 ΔKRAB | KpnI restriction PCR fragment from GAL4DBD-ZFP819 | TTAGGTACCGAGGCAGAGCATGACTCACA, TTAGGTACCTCCCAGTCTCTGGAGCTTCT |
| GAL4DBD-ZFP568 ΔKRAB1 KRAB2MLD-MLE | SD mutagenesis from pcDNA3.1-GAL4DBD-ZFP568 ΔKRAB1 | CGAGCTGTGATGTTGGAGAACTACAGCAACCTGCT , AGCAGGTTGCTGTAGTTCTCCAACATCACAGCTCG |
| Flag-TRIM28 | EcoRI-XbaI digest from GAL4DBD-TRIM28 into pcDNA3.1-Flag vector | |
| pGADT7-TRIM28 | EcoRI restriction PCR-fragment from cDNA clone into pGADT7 vector (Clontech) | TTAGAATTCTTGCGTGATAGTGGCAGTAAGG, TAAGAATTCTGGTTCTACCAGCACAGCAG |
| pGBKT7-ZFP568 WT | EcoRI restriction PCR fragment from cDNA clone into pGBKT7 vector | AATTGTCGACACCCAGCCTTGAAATACCAG, AATTGTCGACTGTTATCCACCACAGGGTTTT |
| pGBKT7-ZFP568 KRAB1 L-P | SD mutagenesis from pGBKT7-ZFP568 WT | GTTGCTGTAGGTCTCCGGCATCACATCTCGGTA, TACCGAGATGTGATGCCGGAGACCTACAGCAAC |
| pGBKT7-ZFP568 KRAB2 L-P | SD mutagenesis from pGBKT7-ZFP568 WT | TCTGTGACCGAGCTGTGATGCCGGATAACTACAGC AACCTG, CAGGTTGCTGTAGTTATCCGGCATCACAGCTCGGT ACAGA |
| pGBKT7-ZFP568 KRAB1 L-P KRAB2 L-P | SD mutagenesis from pGBKT7-ZFP568 KRAB1 L-P | TCTGTGACCGAGCTGTGATGCCGGATAACTACAGC AACCTG, CAGGTTGCTGTAGTTATCCGGCATCACAGCTCGGT ACAGA |
| pGBKT7-ZFP568 ZF | EcoRI restriction PCR fragment from cDNA clone into pGBKT7 vector | AATTGTCGACGTAAGGCAAAGGCCAGTGAG, AATTGTCGACTGTTATCCACCACAGGGTTTT |
| pGBKT7-ZFP568 KRAB1 DV-AA | EcoRI digest from pcDNA3.1-GAL4DBD-ZFP568 KRAB1DVAA into pGBKT7 | |

| | | |
|---|---|---|
| pGBKT7-ZFP568 KRAB2 DV-AA | EcoRI digest from pcDNA3.1-GAL4DBD-ZFP568 KRAB2DVAA into pGBKT7 | |
| pGBKT7-ZFP568 ΔKRAB2 | EcoRI digest from pcDNA3.1-GAL4DBD-ZFP568 KRAB1-ZF into pGBKT7 | |
| pGBKT7-ZFP568 ΔKRAB1 | EcoRI digest from pcDNA3.1-GAL4DBD-ZFP568 KRAB2-ZF into pGBKT7 | |
| pGBKT7-ZFP568 KRAB1 KRAB1 | EcoRI digest from pcDNA3.1-GAL4DBD-ZFP568 KRAB1KRAB1-ZF into pGBKT7 | |
| pGBKT7-ZFP568 KRAB2 KRAB2 | EcoRI digest from pcDNA3.1-GAL4DBD-ZFP568 KRAB2KRAB2-ZF into pGBKT7 | |
| pGBKT7-ZFP57 WT | EcoRI digest from pcDNA3.1-GAL4DBD-ZFP57 WT into pGBKT7 vector | |
| pGBKT7-ZFP57 KRAB S-P | EcoRI digest from pcDNA3.1-GAL4DBD-ZFP57 KRAB S-P into pGBKT7 vector | |
| pGBKT7-ZFP809 WT | EcoRI digest from pcDNA3.1-GAL4DBD-ZFP809 WT into pGBKT7 vector | |
| pGBKT7-ZFP809 KRAB L-P | EcoRI digest from pcDNA3.1-GAL4DBD-ZFP809 KRAB L-P into pGBKT7 vector | |
| pGBKT7-ZFP69 WT | EcoRI restriction PCR fragment from cDNA clone into pGBKT7 vector | TTAGAATTCTTGGACATTGCCGTGGACTTAT, TAAGAATTCTCAAGGTTACTCGAGTCCTGAA |
| pGBKT7-ZFP617 WT | NotI restriction PCR fragment from cDNA clone into pGBKT7 vector | TTAGCGGCCGCTCTGTTCCGGAGCCTCTGTG, TAAGCGGCCGCTCTTTCCAGTGTCAGCTATGTCA |
| pGBKT7-ZFP446 WT | EcoRI restriction PCR fragment from cDNA clone into pGBKT7 vector | TTAGAATTCTTCCCTCTGTCACCTCCAAAAA, TAAGAATTCATGGGCACCTTTTGGTAGAA |
| pGBKT7-ZFP496 WT | NotI restriction PCR fragment from cDNA clone into pGBKT7 vector | TTAGCGGCCGCTCCATCAAATCCCATCATGC, TAAGCGGCCGCCCTGGGTTTTGGCACA |
| pGBKT7-ZFP68 WT | NotI restriction PCR fragment from cDNA clone into pGBKT7 vector | TTAGCGGCCGCAGGACGCTCCATGTCCTG, TAAGCGGCCGCAATCACAGGGTCCC |

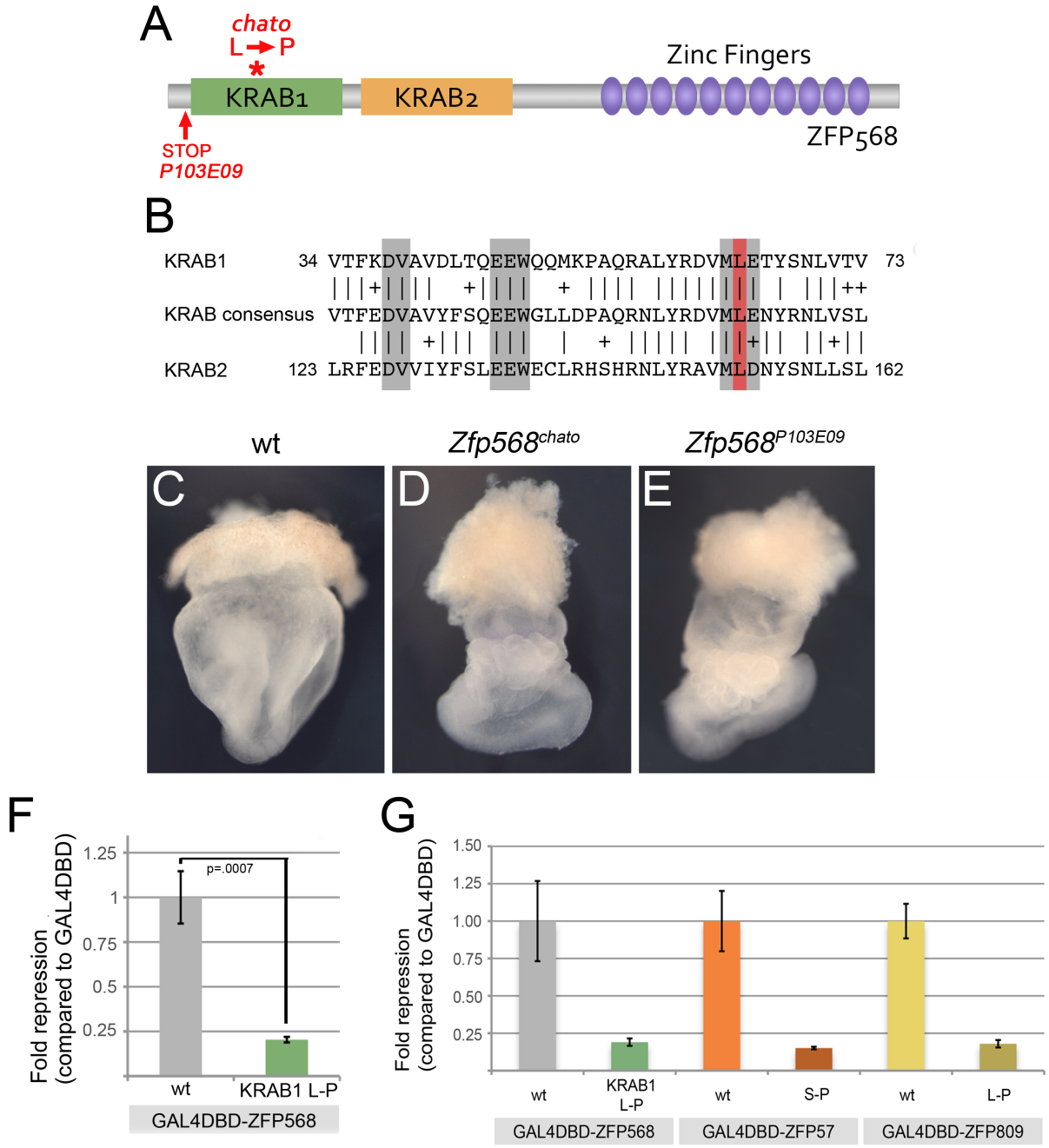
| | | |
|---|---|---|
| pGBKT7-ZFP112 WT | EcoRI restriction PCR fragment from cDNA clone into pGBKT7 vector | TTAGAATTCTTCCTTGTTCCGTTCCTTTCAG, TAAGAATTCGTTCTTGGTTCTCCGCCATA |
| pGBKT7-ZFP61 WT | NotI restriction PCR fragment from cDNA clone into pGBKT7 vector | TTAGCGGCCGCACCTCCTGGAAGGAAGGGTA, TAAGCGGCCGCCGAGTCTTTTCAAGCACTATTTT |
| pGBKT7-ZFP819 WT | NotI restriction PCR fragment from cDNA clone into pGBKT7 vector | TTAGCGGCCGCTTACATGGCTGCTGACATGAAT, TAAGCGGCCGCTGAGCAAACAACTGCACA |
| pGBKT7-ZFP13 WT | EcoRI restriction PCR fragment from cDNA clone into pGBKT7 vector | TTAGAATTCTTGCTGTTTTTCCTGGCTCT, TAAGAATTCCCTGGGTGTTCTCACTGCTC |
| pGBKT7-ZFP568 ΔKRAB2 L-P | SD mutagenesis from pGBKT7-ZFP568 ΔKRAB2 | GTTGCTGTAGGTCTCCGGCATCACATCTCGGTA, TACCGAGATGTGATGCCGGAGACCTACAGCAAC |
| pGBKT7-ZFP110 WT | NotI restriction PCR fragment from cDNA clone into pGBKT7 vector | TTAGCGGCCGCCAACCTCTGGAACCCAGTGT, TTAGCGGCCGCGTGGTGTCCTGAGCTGTCCT |
| pGBKT7-ZFP110 KRAB1 M-P | SD mutagenesis from pGBKT7-WT ZFP110 | CCTGTACAGGGAGATCATGCCGGAGAACTACAGC AACATG, CATGTTGCTGTAGTTCTCCGGCATGATCTCCCTGTA CAGG |
| pGBKT7-ZNF560 WT | NotI restriction PCR fragment from cDNA clone into pGBKT7 vector | TTAGCGGCCGCTTCGTGTTCGGGTCTTTCTAGG, TTAGCGGCCGCAGGCTTGAGGAAACAGCAAA |

RESULTS

**ZFP568 repressor activity is disrupted by *chato* mutation**

The *chato* mutation causes a leucine to proline (L-P) amino acid substitution in the first KRAB motif of ZFP568 (Garcia-Garcia et al., 2008) (Fig. 3.1A, red star B, red highlight). Homozygous *chato* mutant embryos were phenotypically indistinguishable from homozygous mutants for *P103E09* (Fig. 3.1, C,D,E), a null allele of *Zfp568,* which causes a truncated ZFP568 protein containing only the first 11 amino acids (Fig. 3.1A, red arrow) (Shibata et al., 2011). This suggests that the *chato* L-P mutation eliminates ZFP568 function *in vivo* (García-García et al., 2008).

To investigate whether the *chato* mutation (KRAB1 L-P) disrupts the repressive activity




**The first and second ZFP568 KRAB domains have different repressive activities**

**Fig. 3.1. *chato* mutation disrupts ZFP568 *in vivo* function and repressive activity.** (A) Scheme depicting *chato* mutation (red star) and *P103E09* gene trap (red arrow) in ZFP568 protein. (B) Comparison of amino acid sequences of KRAB1 and KRAB2 domains of ZFP568, highlighting residues shown to be important for other KRAB domain protein repressive activities in Margolin et al., 1994 (gray) and the amino acid mutated in *chato* mutants. (C-E) Images of wild type (C), ZFP568$^{chato}$ (D) and ZFP568$^{P103E09}$ (E) embryos dissected at E8.5. (F-G) Quantification of luciferase expression from a 5xUAS-luciferase reporter in HEK293T cells in the presence of wild type or KRAB1 L-P constructs of GAL4DBD-ZFP568 (F), GAL4DBD-ZFP57, or GAL4DBD-ZFP809 (G). Luciferase expression is plotted as fold repression relative to GAL4DBD empty vector, and normalized to wild type conditions (1.0). Error bars represent s.d. One tailed t-test was used to calculate p-values. Panels A-E were contributed by Maho Shibata, and Angela Pring-Mill contributed to data in panel G.

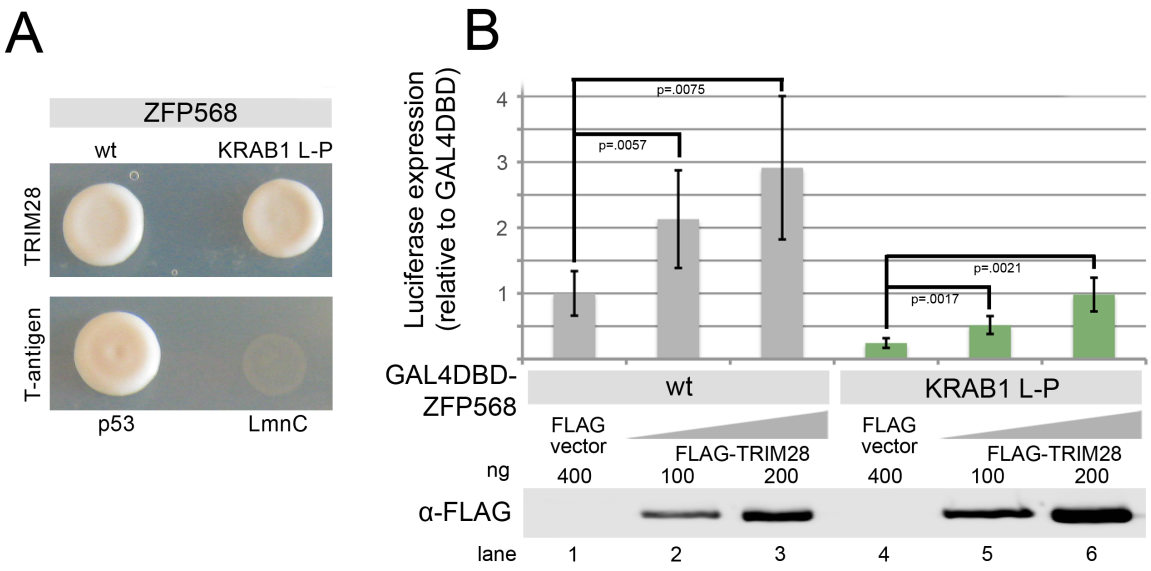



**Fig. 3.2. KRAB1 L-P mutation does not disrupt ZFP568 interaction with TRIM28.** (A) Yeast two-hybrid assays showing interaction of GAL4DBD-ZFP568 (wild type and KRAB1 L-P) with GAL4AD-TRIM28, as indicated by growth on Ade-, His-, Leu-, Trp- media (top panel). p53 interaction with SV40 large T-antigen was used as a positive control and Lamin C (LmnC) interaction with SV40 large T-antigen was used as a negative control (bottom panel). (B) Quantification of luciferase expression from a 5xUAS-luciferase reporter in HEK293T cells in the presence of wild type (gray bars) or KRAB1 L-P (green bars) constructs of GAL4DBD-ZFP568 in the presence of empty vector (lanes 1 and 4) or increasing amounts of Flag-TRIM28 (lanes 2,3,5,6). Luciferase expression is plotted as fold repression relative to GAL4DBD empty vector (1.0). Error bars represent s.d. One tailed t-test was used to calculate p-values.

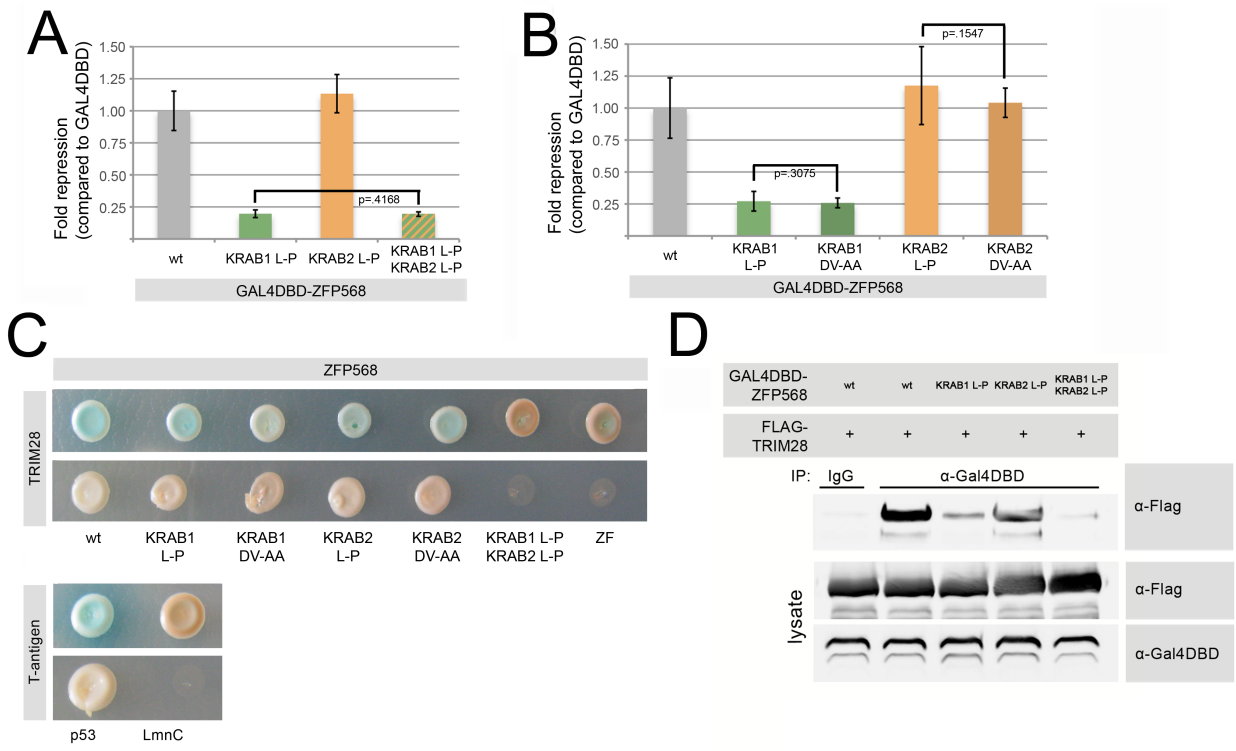



**Fig. 3.3: Differential effects of mutations in the first and second KRAB domains of ZFP568.** (A-B) Quantification of luciferase expression from a 5xUAS-luciferase reporter in HEK293T cells (Fig. 3.1C, D). (A) Fold repression relative to GAL4DBD empty vector, and normalized to wild type conditions (1.0). Error bars represent s.d. (C) Yeast two-hybrid assays showing interaction of wild type and mutated constructs of GAL4DBD-ZFP568 with GAL4AD-TRIM28, as indicated by blue colony color in Leu-, Trp-, X-alpha-gal plates (top panel) and growth on Ade-, His-, Leu-, Trp- media (bottom panel). A construct containing only ZFP568 ZF domains did not show interaction with TRIM28. p53 interaction with SV40 large T-antigen was used as a positive control and Lamin C (LmnC) interaction with SV40 large T-antigen was used as a negative control. (D) Flag-TRIM28 and wild type, KRAB1L-P, and KRAB2L-P constructs of GAL4DBD-ZFP568 co-immunoprecipitate (co-IP) when transfected in HEK293T cells and anti-GAL4DBD antibody was used to immunoprecipitate. Natalia Shylo contributed to data in (A), and Angela Pring-Mill contributed to data in (B). One tailed t-test was used to calculate p-values.

(Fig. 3.3C). However, simultaneous mutations in the first and second KRAB motifs of ZFP568 severely disrupted TRIM28 binding in both yeast two-hybrid and co-immunoprecipitation experiments (Fig. 3.3C, D). The fact that the mutated constructs of GAL4DBD-ZFP568 disrupted only when both KRAB domains are mutated together supports the possibility that both the first and second KRAB domains are capable of binding to TRIM28.

**ZFP568 KRAB1 domain maintains repressive activity regardless of position**

Since mutations in each of the two KRAB domains of ZFP568 did not abolish its interaction with TRIM28, I sought to determine the cause of repressive activity differences between the two domains. The amino acid sequences of both ZFP568 KRAB domains do not differ significantly from the KRAB motif consensus sequence (Fig. 3.1B). To explore whether

the differences between ZFP568 KRAB1 and KRAB2 repressive activities are due to their relative position within the protein, I tested the repressive activities of mutants lacking either the first or second KRAB domain. A ZFP568 protein lacking the second KRAB domain (ΔKRAB2) maintained repressive activity comparable to wild type ZFP568, while the repressive activity of a protein lacking the first KRAB domain (ΔKRAB1) was significantly reduced (Fig. 3.4A, dark green and orange bars). Therefore, the position of the first and second KRAB domains within the protein does not influence ZFP568 repressive activity. Additionally, an engineered ZFP568 protein containing two KRAB2 domains did not show significant repressive activity compared with an engineered protein containing two KRAB1 domains (Fig. 3.4A, green and orange plaid). These results demonstrate that neither the number nor relative position of KRAB domains within ZFP568 influence its repressive activity, and suggest that perhaps intrinsic differences in the amino acid composition of each KRAB domain are important for KRAB mediated repression.

ZFP568 proteins containing KRAB1 motifs were able to bind TRIM28, but ZFP568 proteins with one or two KRAB2 motifs that lacked the KRAB1 motif (ΔKRAB1 and KRAB2-KRAB2 ZF) failed to recruit TRIM28 (Fig. 3.4B). Therefore, the second KRAB domain of ZFP568 does not bind to TRIM28 or repress transcription in the absence of the first KRAB domain. This result seems to conflict with my previous finding that TRIM28 binding to ZFP568 is only abolished when both of its KRAB domains contain L-P mutations (Fig. 3.3C, D). However, I found that a mutant ZFP568 containing the KRAB1 L-P mutation and lacking the second KRAB domain (ΔKRAB2 KRAB1 L-P) was unable to bind TRIM28 (Fig. 3.4C), suggesting that the L-P mutation completely abolishes TRIM28 binding to the KRAB motif. In view of these results, I conclude that an intact second KRAB domain in ZFP568 can somehow

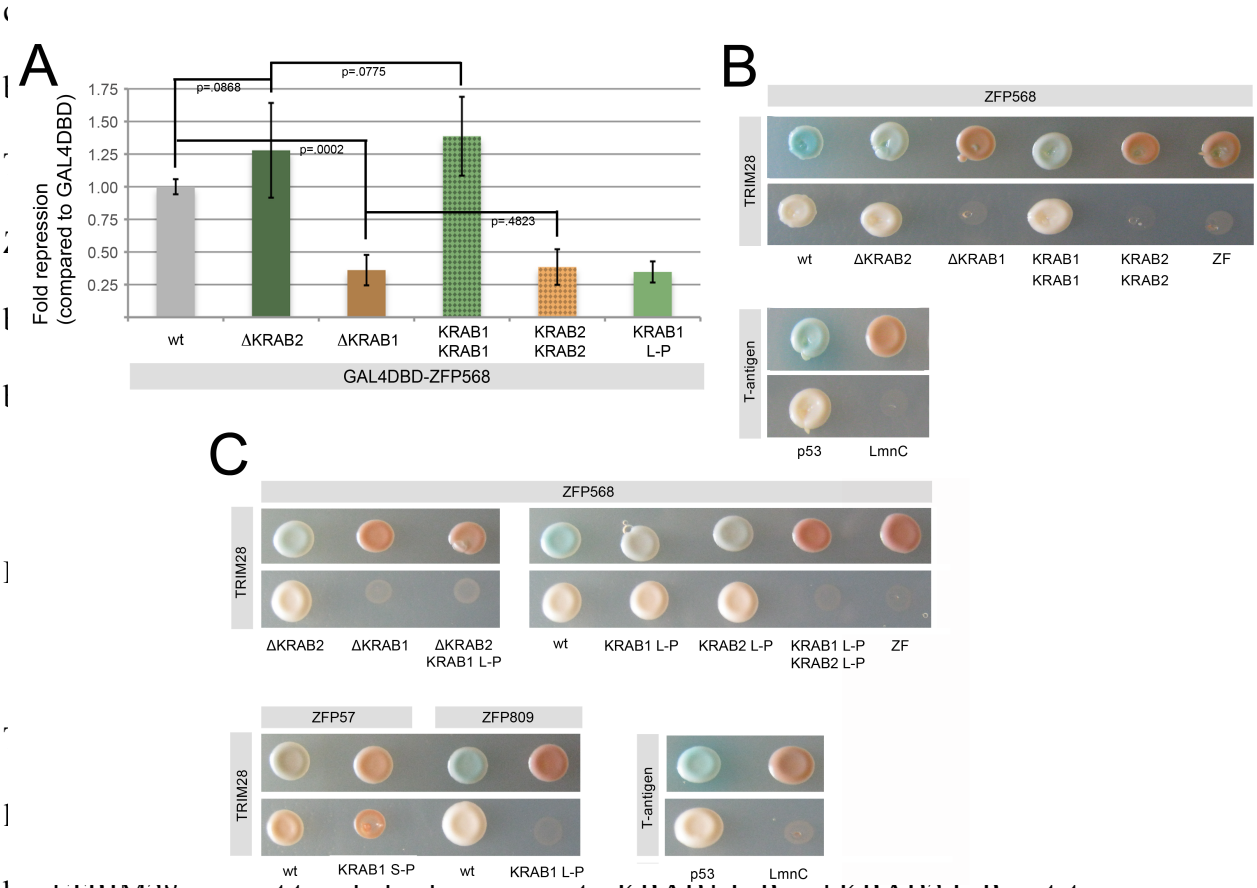
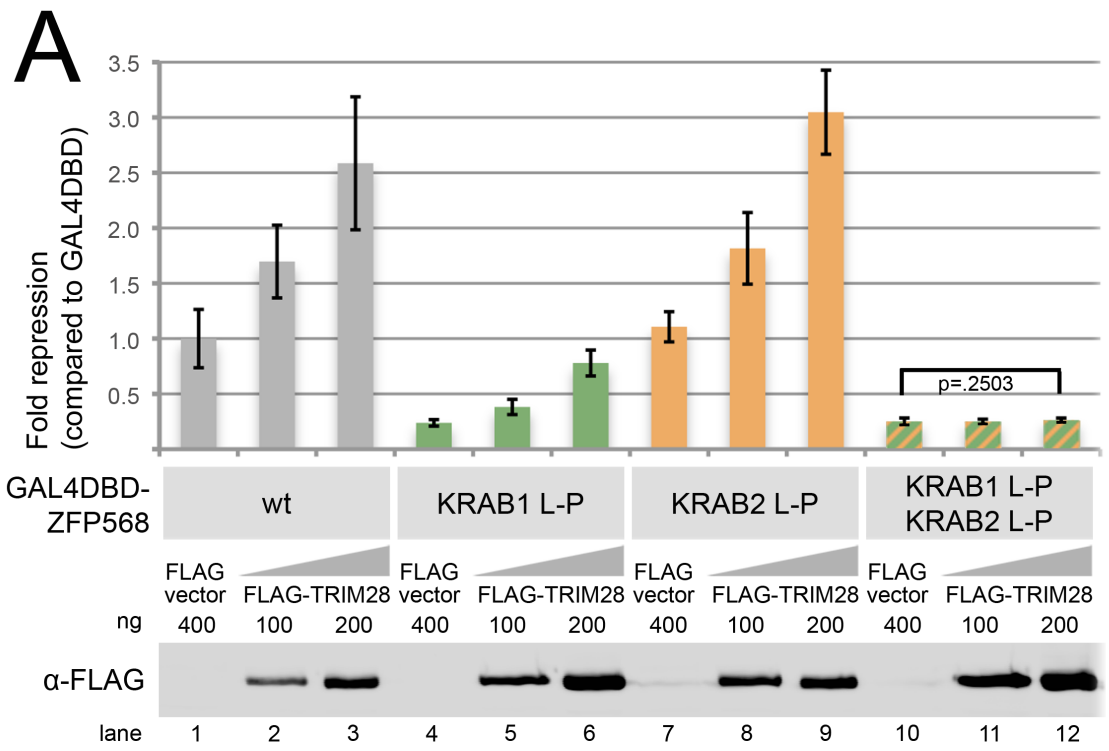



**Fig. 3.4. The repressive activity of ZFP568 depends on amino acid sequence of KRAB motifs.** (A) Quantification of luciferase expression in HEK293T cells, in the presence of wild type (gray bar), ΔKRAB2 (dark green bar), ΔKRAB1 (dark orange bar), or KRAB1 KRAB1 (green plaid bar), KRAB2 KRAB2 (orange plaid bar), or KRAB1 L-P (light green bar) constructs of GAL4DBD-ZFP568. Luciferase expression is plotted as fold repression relative to GAL4DBD empty vector, and normalized to wild type conditions (1.0). Error bars represent s.d. (B-C) Yeast two-hybrid assays showing interaction of wild type and mutated constructs of GAL4DBD-ZFP568 with GAL4AD-TRIM28, as indicated by blue colony color in Leu-, Trp-, X-alpha-gal plates (top panels) and growth on Ade-, His-, Leu-, Trp- media (bottom panels). p53 interaction with SV40 large T-antigen was used as a positive control and Lamin C (LmnC) interaction with SV40 large T-antigen was used as a negative control. Cecilia Copperman contributed to data in (A). One tailed t-test was used to calculate p-values.

bind TRIM28 in yeast two-hybrid experiments, KRAB1 L-P and KRAB2 L-P mutations in ZFP568 showed enhanced repressive activity in response to increasing amounts of TRIM28 (Fig. 3.5A). These results further support that the KRAB1 L-P mutation did not abolish ZFP568 binding to TRIM28 when the second KRAB domain is present. I also investigated ZFP568 repressive activity following depletion of endogenous TRIM28. When TRIM28 protein levels were reduced by siRNA, wild type ZFP568 and ZFP568$^{KRAB2LP}$ repressive activities were severely disrupted (Fig. 3.5B), implying that ZFP568 represses transcription through TRIM28-dependent mechanisms. Interestingly, ZFP568$^{KRAB1LP}$ and ZFP568$^{KRAB1LPKRAB2LP}$ repressive activities were not affected (Fig. 3.5B), suggesting that the leucine residue in the first KRAB domain of ZFP568 is critical for TRIM28-dependent repression. The fact that full length ZFP568 containing the KRAB1 L-P mutation interacts with TRIM28, but lacks repressive activity


A
Fold repression (compared to GAL4DBD)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
p=.2503
GAL4DBD-ZFP568
wt
KRAB1 L-P
KRAB2 L-P
KRAB1 L-P KRAB2 L-P
FLAG vector
FLAG-TRIM28
ng
400
100
200
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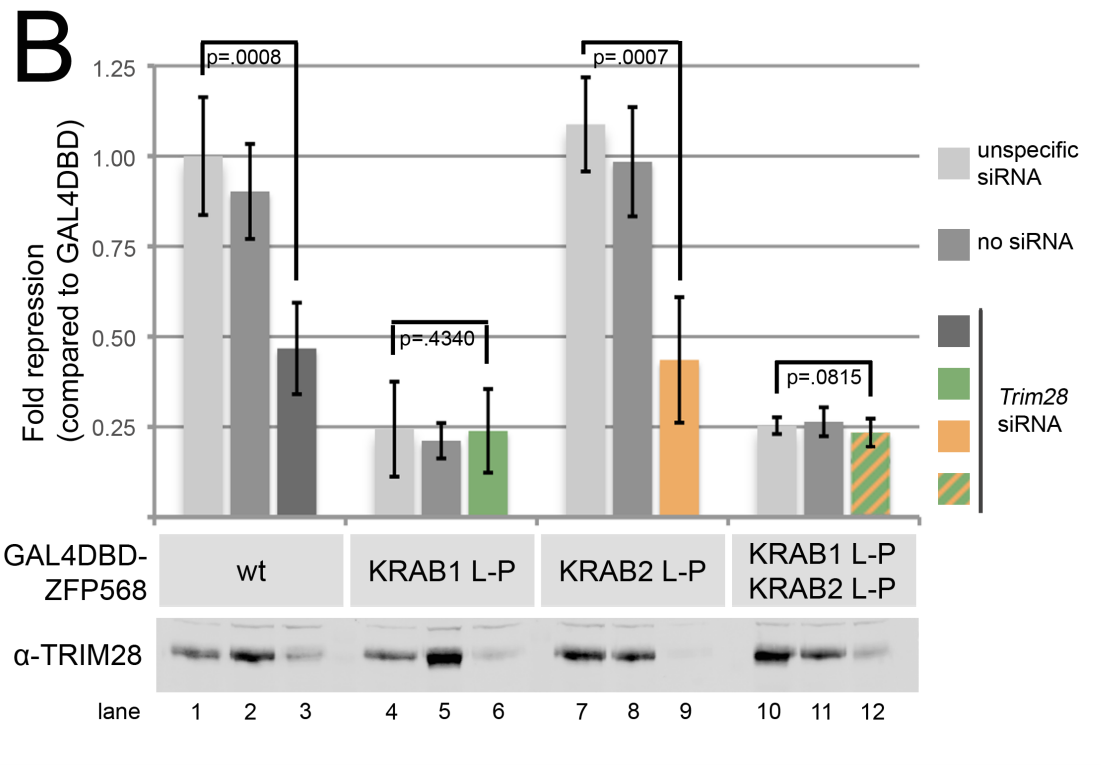
1 2 3 4 5 6 7 8 9 10 11 12


B
Fold repression (compared to GAL4DBD)
1.25
1.00
0.75
0.50
0.25
p=.0008
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p=.0007
p=.0815
unspecific siRNA
no siRNA
Trim28 siRNA
GAL4DBD-ZFP568
wt
KRAB1 L-P
KRAB2 L-P
KRAB1 L-P KRAB2 L-P
α-TRIM28
lane
1 2 3 4 5 6 7 8 9 10 11 12

**Fig. 3.5. Mutations in the first and second KRAB domains differentially affect ZFP568 response to TRIM28.** (A) Quantification of luciferase expression in HEK293T cells, in the presence of wild type (gray bars), KRAB1L-P (green bars), KRAB2 L-P (orange bars), or KRAB1L-P KRAB2L-P (orange/green striped bars) constructs of GAL4DBD-ZFP568 and presence of empty vector (lanes 1,4,7,10) or increasing amounts of FLAG-TRIM28 (lanes 2,3,5,6,8,9,11,12). Western blots show levels of FLAG-TRIM28 protein normalized for transfection efficiency. (B) Quantification of luciferase expression in HEK293T cells, in the presense of wild type (lanes 1-3), KRAB1L-P (lanes 4-6), KRAB2 L-P (lanes 7-9), or KRAB1L-P KRAB2L-P (lanes 10-12) constructs of GAL4DBD-ZFP568 with (colored bars) and without (gray bars) treatment with *Trim28* siRNA. Western blots show levels of FLAG-TRIM28 protein normalized for transfection efficiency. Luciferase expression is plotted as fold repression relative to GAL4DBD empty vector, and normalized to wild type conditions (1.0). Error bars represent s.d. One tailed t-test was used to calculate p-values.

indicates that an interaction with TRIM28 is not sufficient for ZFP568 KRAB domain repressive activity.

**Additional KRAB Zinc Finger proteins possess less repressive KRAB domains**

To determine whether the repressive properties of ZFP568 typify members of the mouse and human KRAB Zinc Finger protein family, I tested the repressive activity and ability to bind TRIM28 of two mouse and human KRAB domain proteins that contain two KRAB motifs. In luciferase assays, repressive activities varied significantly among different family members (Fig. 3.6A). Because only the first KRAB domain of ZFP568 repressed transcription in our luciferase assays, we investigated whether one or both KRAB domains of zinc finger protein 110 (ZFP110) and human zinc finger protein 568 (ZNF568) could repress transcription. When the first KRAB motif of ZFP110 was mutated in the analogous residue to that affected by the *chato* mutation (M-P substitution), its repressive activity was substantially reduced (Fig. 6B, dark pink bar compared to gray bar). However, similar to ZFP568, the repressive activity of ZFP110 was unaffected by an analogous L-P mutation in its second KRAB domain (Fig. 3.6B, light pink bar compared to gray bar). This suggests that similar to ZFP568, the KRAB motifs of ZFP110 have different repressive activities. To determine whether these KRAB motifs also have different abilities to bind TRIM28, I used yeast two-hybrid assays. ZFP110$^{KRAB1MP}$ retained the ability to interact with TRIM28 (Fig. 3.6B), suggesting that, similar to ZFP568, the second ZFP110 KRAB motif can still bind TRIM28 and/or compensate for the KRAB1 M-P mutation. In contrast to ZFP110, human ZNF568 retained repressive activity when either the first or second KRAB domains contained the corresponding L-P mutation (Fig. 3.6C, dark and light green bars compared to gray

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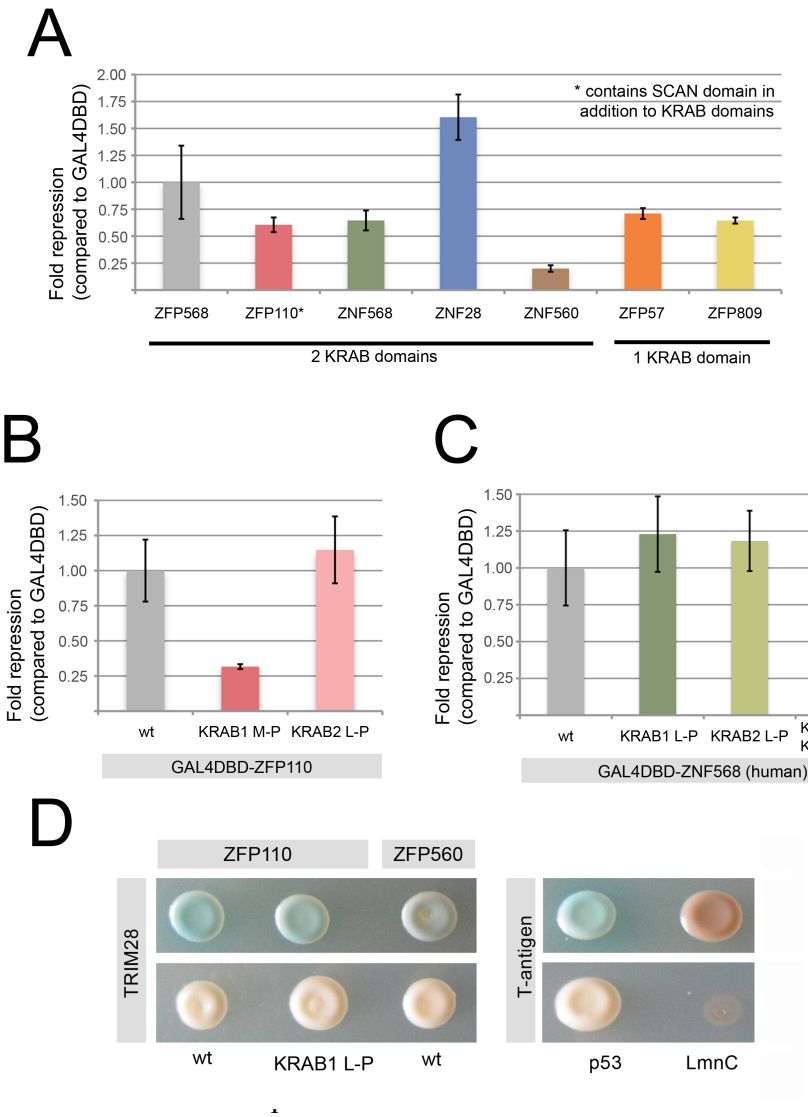


**Fig. 3.6. Repressive activity of mouse and human KRAB domain proteins other than ZFP568.** (A) Quantification of luciferase expression in HEK293T cells, in the presence of constructs of various GAL4DBD-KRAB-Zinc Finger proteins. (B-C) Quantification of luciferase expression in HEK293T cells, in the presence of wild type (gray bar), KRAB1 L-P (dark pink bar), or KRAB2 L-P (light pink bar) constructs of GAL4DBD-ZFP110 (B) or wild type (gray bar), KRAB1 L-P (dark green bar), KRAB2 L-P (light green bar), or KRAB1 L-P, KRAB2 L-P constructs of GAL4DBD-ZNF568 (C). Luciferase expression is plotted as fold repression relative to GAL4DBD empty vector, and normalized to wild type conditions (1.0). Error bars represent s.d. (D) Yeast two-hybrid assays showing interaction of wild type and mutated constructs of GAL4DBD-ZFP110 or GAL4DBD-ZNF560 with GAL4AD-TRIM28, as indicated by blue colony color in Leu-, Trp-, X-alpha-gal plates (top panels) and growth on Ade-, His-, Leu-, Trp- media (bottom panels). p53 interaction with SV40 large T-antigen was used as a positive control and Lamin C (LmnC) interaction with SV40 large T-antigen was used as a negative control.

not due to their relative positions within the protein (Fig. 3.4A), I hypothesized that differences in the amino acid sequence within the KRAB motifs could be important in determining their repressive activities (Fig. 3.7A, yellow and blue highlights). To explore this, I introduced amino acid changes into the first KRAB motif of ZFP568 (using the AKRAB2 construct) to make it resemble the second KRAB domain, and tested whether any of these changes could reduce the strong repressive activity of this motif. I found that a subset of the substitutions had a partial effect on repressive activity (Fig. 3.7B, blue bars), while others did not have any effect (Fig. 3.7B, yellow bars). I also performed reciprocal experiments with one of the residues to introduce

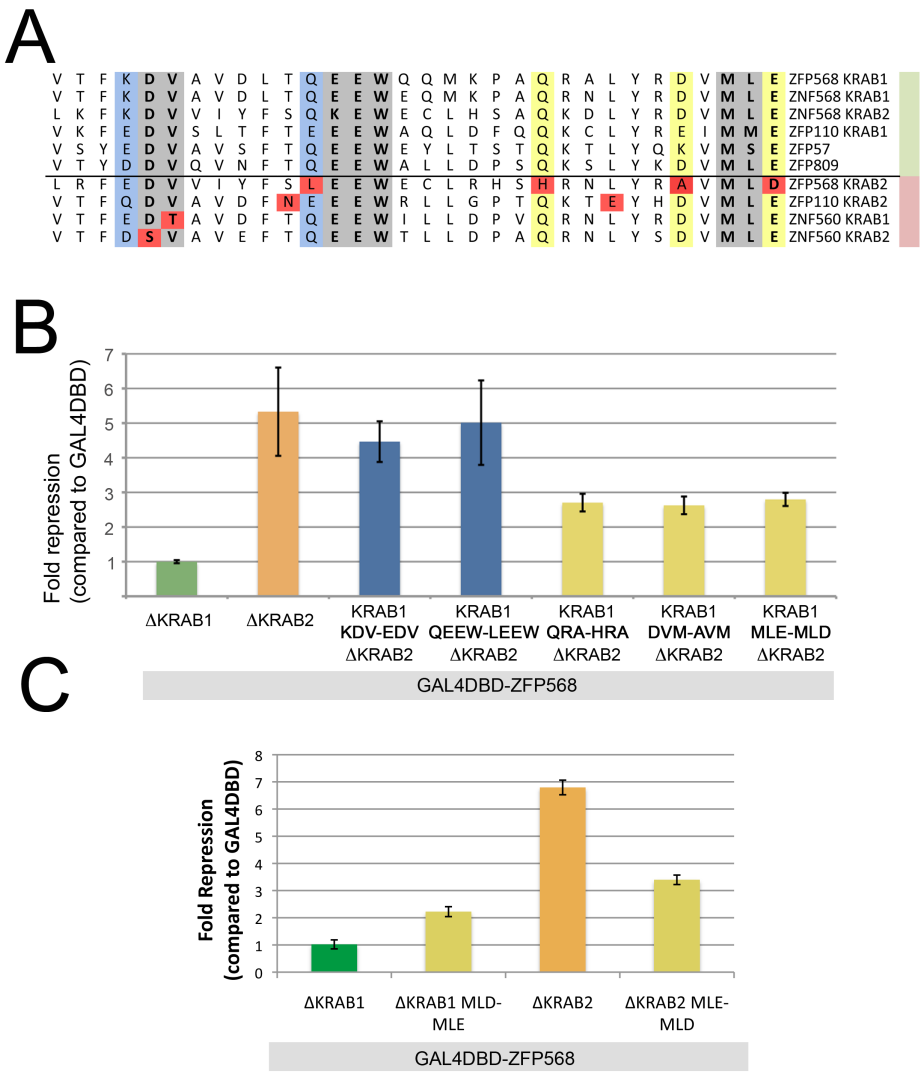


**Fig. 3.7. Differences in the amino acid sequence of the KRAB motif are responsible for the different repressive activities amongst KRAB domain proteins.** (A) Sequence comparison of KRAB motifs of the indicated proteins. Sequences above line represent KRAB motifs with highly efficient repressive activities (green), and sequences below line represent KRAB motifs with less efficient repressive activities (red). Red highlighted residues represent substitutions in conserved amino acids in less repressive KRAB motifs. Residues previously shown to be important for repressive activity by Margolin et al., 1994 are highlighted in gray. Conserved residues shown here to be important for ZFP568 repressive activity are highlighted in yellow, and conserved residues with no effect on repressive activity are highlighted in blue. (B-C) Quantification of luciferase expression in HEK293T cells, in the presence of ΔKRAB1 (green bar), ΔKRAB2 (orange bar) and mutated constructs of ΔKRAB2 (B) and ΔKRAB1 (C). Yellow blue bars highlight mutations that do not affect repressive activity, and yellow bars highlight mutations that partially affect repressive activity. Luciferase expression is plotted as fold repression relative to GAL4DBD empty vector, and normalized to wild type conditions (1.0). Error bars represent s.d.

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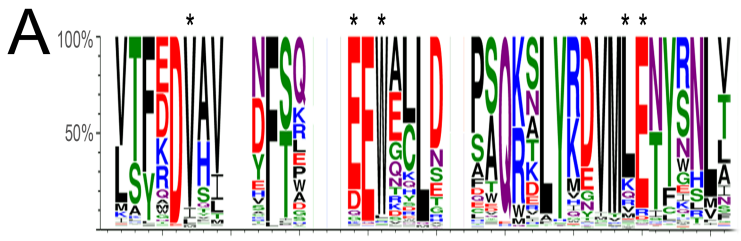
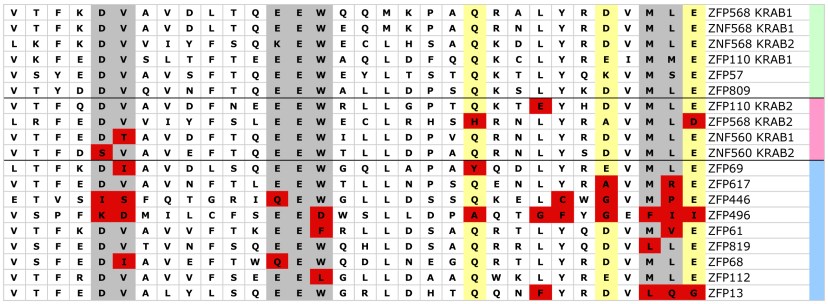
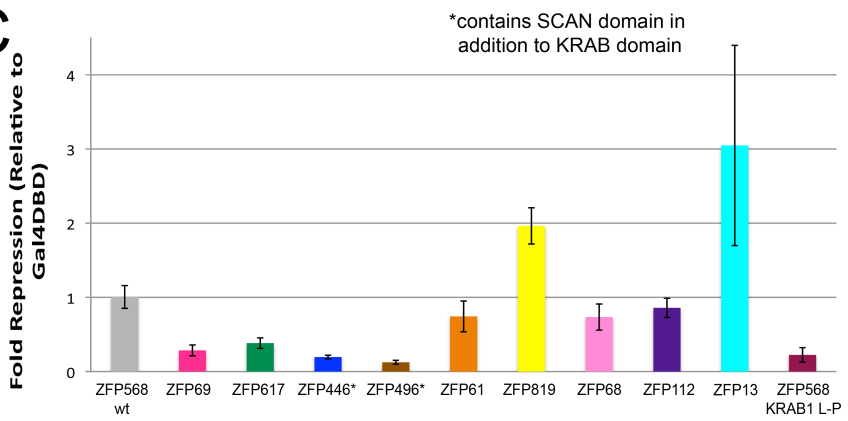
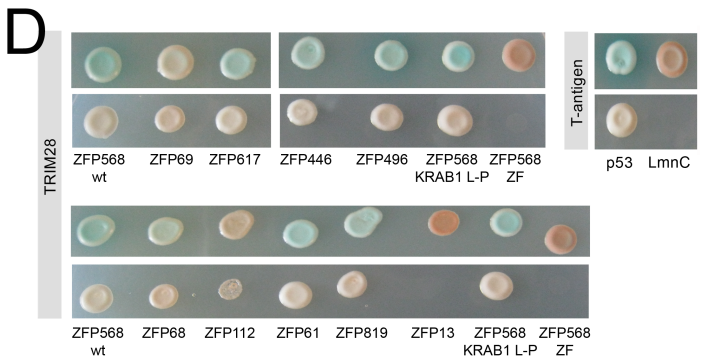


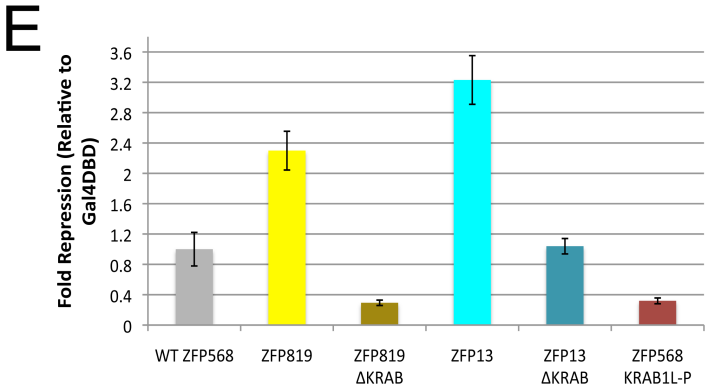



**Figure 3.8. KRAB domain repressive activity does not always correlate with ability to interact with TRIM28.** (A) Weblogo representing the probability of each amino acid residue within the KRAB motif for all mouse KRAB motifs. Stars represent amino acids found to affect repressive activity with a substantial number of KRAB domains containing an amino acid substitution from the most conserved. (B) Sequence comparison of KRAB motifs of the indicated proteins. Sequences in top section represent represent KRAB motifs with highly efficient repressive activities (green), sequences in the middle section represent KRAB motifs with less efficient repressive activities (red), and sequences in the bottom section represent sequences chosen for analysis with substitutions in conserved amino acids (red highlights). Residues previously shown to be important for repressive activity by Margolin et al., 1994 are highlighted in gray. Conserved residues shown here to be important for ZFP568 repressive activity are highlighted in yellow. (C) Quantification of luciferase expression in HEK293T cells, in the presence of constructs of various GAL4DBD-KRAB Zinc Finger proteins. (D) Yeast two-hybrid assays showing interaction different GAL4DBD KRAB Zinc Finger protein constructs with GAL4AD-TRIM28, as indicated by blue colony color in Leu-, Trp-, X-alpha-gal plates (top panels) and growth on Ade-, His-, Leu-, Trp- media (bottom panels). p53 interaction with SV40 large T-antigen was used as a positive control and Lamin C (LmnC) interaction with SV40 large T-antigen was used as a negative control. (E) Quantification of luciferase expression in HEK293T cells, in the presence of constructs of various wild type and mutated constructs of GAL4DBD-KRAB Zinc Finger proteins. Luciferase expression is plotted as fold repression relative to GAL4DBD empty vector, and normalized to wild type conditions (1.0). Error bars represent s.d.

predicted, I observed that KRAB Zinc Finger proteins containing amino acids that diverged at critical KRAB residues, including ZFP69, ZFP617, ZFP446, and ZFP496, all had a poor repressive activity (Fig. 3.8C). However, some proteins with amino acid substitutions in residues important for KRAB-mediated repression, such as ZFP61, ZFP819, ZFP68, ZFP112, and ZFP13, could efficiently repress transcription. (Fig. 3.8C).

In order to determine whether these KRAB Zinc Finger proteins require TRIM28 binding for transcriptional repression, I assayed the ability of the same set of proteins to bind TRIM28 in yeast two-hybrid assays (Fig. 3.8D). Surprisingly, the repressive activity of these KRAB domain proteins did not correlate with their ability to recruit TRIM28. Some KRAB Zinc Finger proteins, like ZFP446 and ZFP496, did not repress transcription, yet interacted with TRIM28 (Fig. 3.8C, D). Together with my analysis of mutant forms of ZFP568, these results provide additional evidence that TRIM28 binding is not sufficient for KRAB mediated repression. Other KRAB Zinc Finger proteins, like ZFP13, could repress transcription, but could not bind to TRIM28 (Fig. 3.8C, D). However, the repressive activity of a mutant form of ZFP13 lacking its KRAB motif (ZFP13$^{\Delta KRAB}$) was impaired, suggesting that its repressive activity is dictated, at least partially, by its KRAB motif and not exclusively by other protein domains. Together these results reveal that TRIM28 is not sufficient for KRAB Zinc Finger proteins to achieve transcriptional repression, and that the repressive activity of KRAB domain proteins might not exclusively depend on TRIM28 but also on other, yet unknown factors.

DISCUSSION

KRAB Zinc Finger proteins are regulators of important and specific processes in mammals. Despite their fundamental roles, relatively little is known about how these proteins function mechanistically. The generally accepted model for all KRAB Zinc Finger family members is that they mediate transcriptional repression through an essential interaction with co-repressor protein TRIM28. However, this model has not been tested extensively for different members of the KRAB Zinc Finger protein family.

**TRIM28 is neither sufficient nor necessary for KRAB domain repression**

The *chato* mutation in the first KRAB domain of ZFP568 was identified in a forward mutagenesis screen in mouse for its effect on embryonic morphogenesis. I found that mutant ZFP568 protein containing the *chato* KRAB1 L-P mutation did not repress transcription in reporter assays, but interestingly, could bind to TRIM28. Therefore, the interaction with TRIM28 is not sufficient to mediate KRAB Zinc Finger repression. While ZFP568$^{KRAB1LP}$ bound to TRIM28 in yeast two-hybrid assays, co-immunoprecipitation results in mammalian cells suggest that the interaction was weakened as compared to wild type ZFP568. Therefore, it is possible that a certain threshold level of TRIM28 recruitment is required for KRAB domains to repress transcription. Another possibility is that factors other than TRIM28 are required in order for KRAB domains to achieve transcriptional repression.

My data also suggest that TRIM28 binding is not always required for KRAB domain protein repressive activity. It has previously been reported that some human KRAB domain proteins that contain both KRAB and SCAN domains repress transcription independent of

TRIM28 (Itokawa et al., 2009). However, some of proteins that I analyzed, including ZFP112 and ZFP13, did not contain SCAN motifs, yet they showed repressive activity in the absence of TRIM28 recruitment. Therefore, it is possible that the KRAB domain of ZFP13 and other proteins have acquired a mechanism to repress transcription independent of TRIM28.

**Evolution of non-repressive KRAB domain proteins**

KRAB Zinc Finger proteins have evolved at high rates (Bustamante et al., 2005; Lorenz et al., 2010), and it has been proposed that the major mechanism of their evolution has been duplication (Hamilton et al., 2006). Therefore, it is possible that KRAB Zinc Finger proteins with two KRAB motifs arose due to a duplication of an exon encoding a KRAB motif, and that during the course of evolution, one of the two KRAB motifs degenerated due to the lack of selective pressure. However, my data indicate that at least in ZFP568, the second KRAB motif can partially rescue the inability to bind TRIM28 caused by mutations in the first KRAB domain. Therefore, it is possible that in some KRAB domain proteins a second KRAB motif evolved accessory functions to enhance the transcriptional repression activity of the first KRAB domain. Thus, organisms with genes encoding proteins with two KRAB domains could possess an evolutionary advantage compared to those with genes encoding just one KRAB domain.

I found that some KRAB Zinc Finger proteins with amino acids divergent from the consensus KRAB domain sequence were weak transcriptional repressors in luciferase expression assays. Similarly to proteins with two KRAB domains, it is possible that single KRAB domain proteins have evolved from duplicated genes and have functions redundant with their ancestor protein, allowing for relaxed evolutionary constraint. This could explain the loss of repressive

activities for some KRAB domain proteins. In this sense, it is possible that non-repressive KRAB Zinc Finger proteins may not be expressed or functional at all. However, several of the KRAB domain proteins with low repressive activities used in my studies, including ZFP69 and ZFP617, and ZFP496, have been implicated in specific biological processes or diseases (Mysliwiec et al., 2007; Scherneck et al., 2009; Wu et al., 2011). In fact, ZFP496 was previously reported to activate transcription, in agreement with my result that it does not repress transcription (Mysliwiec et al., 2007). Therefore, I favor a hypothesis in which non-repressive KRAB domain proteins, instead of being degenerate and non-functional, accumulated mutations in residues critical for transcriptional repression that allowed the evolution of new functions for these DNA binding proteins.

Taken together, my findings challenge the assumption that TRIM28 is required and sufficient for KRAB mediated repression, and provide evidence that mammalian genomes contain KRAB domain proteins with different levels of transcriptional repression activity.

ACKNOWLEDGEMENTS

I thank undergraduate students Natalia Shylo, Angela Pring-Mill, Cecilia Copperman for working with me on this project. I thank Drs. John Schimenti, Mariana Wolfner, and members of the laboratory for helpful discussions. I also thank Drs. Muriel Aubry, Xavier Mascle, and Ling Qi for equipment and reagents.

# CHAPTER 4

# EXPANDED DISCUSSION AND FUTURE DIRECTIONS

The functions of the vast majority of KRAB Zinc Finger proteins have not yet been determined, and our current knowledge about the repressive mechanisms utilized by the KRAB Zinc finger family remains limited. This dissertation aimed to elucidate the molecular roles of KRAB Zinc Finger protein ZFP568 in concert with TRIM28, and to extend these findings to a larger sample of KRAB Zinc Finger proteins in order to gain insight about the family as a whole.

**Differential requirement for TRIM28 by KRAB Zinc Finger proteins**

The results presented in this thesis showed that TRIM28 physically interacts with ZFP568 and is required for ZFP568 mediated transcriptional repression. I found that the hypomorphic *chatwo* allele of *Trim28* encodes a protein that is less efficient than wild type TRIM28 in repressing transcription, and causes protein instability for TRIM28-KRAB Zinc Finger complexes (Chapter 2). My results suggest that ZFP568 possesses a higher requirement for TRIM28 than other KRAB Zinc Finger proteins, which explains the phenotypic similarities between *chatwo* embryos and *chato* mutants in *Zfp568*. (Chapter 2) While my data showed that the repressive activity of ZFP568 was the most severely affected by RNAi reduction of TRIM28 level, the repressive activities of both ZFP57 and ZFP809 were reduced by depletion of TRIM28. In support of this, data obtained in the Garcia-Garcia laboratory by Maho Shibata and Kate Alexander have indicated that the *chatwo* mutations in TRIM28 affect the functions of two other KRAB Zinc Finger proteins, ZFP57 and ZFP809, in genomic imprinting and ES cell retroviral silencing (Shibata and Alexander, unpublished data; Shibata et al., 2011).

Assuming that more than 400 KRAB Zinc Finger proteins encoded in the mouse genome require TRIM28 for their functions, it is not surprising that depletion of TRIM28 results in early

embryonic arrest at E5.5 (Cammas et al., 2000). However, partial depletion of TRIM28 function and levels by the *chatwo* mutations allow for embryonic survival until E9 (Shibata et al., 2011). I hypothesize that the interaction with one or more KRAB Zinc Finger protein is responsible for the early arrest of *Trim28* null embryos at E5.5. To test this model, one may be able to identify KRAB Zinc Finger protein(s) that function at these earlier time points, and to investigate their requirements for TRIM28.

In addition, studies should be aimed at better understanding the full profile of KRAB Zinc Finger proteins required during mouse development. KRAB Zinc Finger proteins important during embryogenesis can be narrowed down based on genome wide expression profiles. Knockout mouse models would be valuable to further understand the roles of individual KRAB Zinc Finger proteins *in vivo*.

Future studies will also be necessary to determine which downstream targets of TRIM28-ZFP568 complexes are involved in regulating embryonic morphogenesis. There are several genome-wide published studies that provide information about TRIM28 targets (O'Geen et al., 2007; Hu et al., 2009; Iyengar et al., 2011). However, similar genome-wide chromatin immunoprecipitation approaches have only been performed for three KRAB Zinc Finger proteins (ZFP263, ZFP274, and ZFP57) and of these, only ZFP57 has been studied for both genomic occupancy and *in vivo* functions (Li et al., 2008; Frietze et al., 2010a, 2010b; Quenneville et al., 2011). It will be interesting to determine the genomic targets of ZFP568 in order to link its regulation of morphogenesis to the expression of specific genes. Microarray based experiments are difficult to analyze due to embryonic tissue specific effects. Additionally, genome wide ChIP experiments are challenging because specific antibodies against ZFP568 are not available due to

the highly homologous nature of KRAB Zinc Finger proteins. Stable integration of tagged KRAB Zinc Finger proteins has also been problematic for our lab and others (Wolf and Goff, 2009; Frietze et al., 2010b; Iyengar and Farnham, 2011). Therefore, it will be of interest to take advantage of advanced expression analyses, such as RNA-seq, in order to better understand the differences in gene expression between wild type and *Zfp568*$^{chato}$ samples, using partial embryos or ES cells. If *Trim28*$^{chatwo}$ samples are also analyzed in these experiments, they may allow one to distinguish ZFP568-dependent and ZFP568-independent processes affected in *chatwo* embryos.

**Not all KRAB domains are efficient repressors**

In addition to my studies about the *chatwo* mutations in TRIM28, I also presented an examination of the effects of the *chato* mutation on ZFP568. I found that the *chato* KRAB1 L-P mutation, which eliminates ZFP568 function *in vivo*, also disrupts ZFP568 transcriptional repression activity, but not its ability to interact with TRIM28. This implies that TRIM28 binding is not sufficient for ZFP568-mediated repression. These findings are significant as they suggest that TRIM28 binding may not be the only factor that determines ZFP568 repressive activity. It is possible that some KRAB Zinc Finger proteins that have lost the ability to interact with TRIM28 through amino acid substitutions, and have gained the ability to repress transcription through other mechanisms. Follow-up studies aimed to identify KRAB domain interacting factors besides TRIM28 are discussed in Appendix A.

Through ZFP568 deletion/mutation studies, I found that while the sequence of the second KRAB domain of ZFP568 does not possess repressive activity or TRIM28 binding ability, it does

compensate for the inability of a mutated first KRAB domain to bind TRIM28. This result suggests that within the KRAB domain, different amino acids or structural features may have different roles in binding TRIM28. Studies aimed to solve the structure of ZFP568 protein or the structure of ZFP568 bound to TRIM28 could elucidate the mechanism of this compensation. Since the KRAB domains in ZFP568 lie adjacent to each other, I hypothesize that different residues or structural features of the first and second RKAB domains may bind cooperatively to different regions of TRIM28.

My analysis indicated that the two KRAB domains of ZFP568 are not functionally equivalent, as the second KRAB domain does not possess repressive activity. I showed that other KRAB Zinc Finger proteins possess qualities similar to ZFP568. For example, some KRAB domains do not possess efficient transcriptional repression activity but maintain an interaction with TRIM28, and some KRAB Zinc Finger proteins repress transcription efficiently but do not interact with TRIM28. This result was unexpected as it contrasts with previously observed correlations between KRAB Zinc Finger repressive activity and TRIM28 binding (Kim et al., 1996; Moosmann et al., 1996). One explanation for this discrepancy is that the proteins I analyzed were specifically selected for their containing less conserved amino acid residues compared to the consensus KRAB sequence.

**Broad implications of KRAB Zinc Finger protein studies**

Recently, several reports have hinted that KRAB Zinc Finger protein and TRIM28 molecular mechanisms are actually more complicated than originally thought (O'Geen et al., 2007; Frietze et al., 2010b; Iyengar and Farnham, 2011). Genome-wide ChIP analyses have

revealed that TRIM28 and one KRAB Zinc Finger, ZNF273, to a large extent occupy the 3' ends of KRAB Zinc Finger proteins (O'Geen et al., 2007; Frietze et al., 2010b). Furthermore, it was shown that when TRIM28 is mutated to perturb binding to KRAB domains, there is no disruption of TRIM28 localization to gene promoters, suggesting that KRAB Zinc Finger proteins may not even function with TRIM28 to regulate transcription (O'Geen et al., 2007; Frietze et al., 2010b; Iyengar and Farnham, 2011; Iyengar et al., 2011). These studies highlight the need for more comprehensive studies aimed to investigate the functions of KRAB Zinc Finger proteins, as well as better knowledge about the *in vivo* targets of KRAB Zinc Finger proteins. Perhaps further genome-wide studies on a variety of cell types or developmental time points could better elucidate when and where KRAB Zinc Finger proteins are required for TRIM28 to localize to gene promoters.

It is important to understand transcriptional regulation of mechanisms in humans. By studying KRAB Zinc Finger proteins, the largest family of transcription factors in mammals, we can determine the mechanisms by which they repress transcription. Furthermore, we may gain valuable information about the regulation of biological processes specific to higher organisms, such as humans, which may be directly or indirectly relevant for clinical applications. The results presented in this dissertation add to our current knowledge of the molecular mechanisms by which KRAB Zinc Fingers function, and have potential applications for understanding embryonic defects, cancer, imprinting defects, obesity, behavioral defects, and other diseases.

# APPENDIX A

# IDENTIFICATION OF NOVEL INTERACTING PARTNERS FOR KRAB ZINC FINGER PROTEIN ZFP568

INTRODUCTION

KRAB domain proteins are thought to function as transcriptional repressors by binding TRIM28, a scaffold protein that recruits chromatin-modifying enzymes (Kim et al., 1996; Moosmann et al., 1996; Nielsen et al., 1999; Ryan et al., 1999; Schultz et al., 2001, 2002). It is not known, however, whether TRIM28 is the only binding partner required for their functions.

As described in chapter 3 of this dissertation, the *chato* mutation disrupts the first of two KRAB domains in ZFP568, and creates a functionally null protein *in vivo* (García-García et al., 2008). We showed that ZFP568 functions with TRIM28 to control embryonic morphogenesis (Shibata et al., 2011). Yet, it is unknown whether additional factors function with ZFP568 during embryogenesis. This led me to hypothesize that ZFP568 interacts with proteins other than TRIM28 to fulfill its functions.

Here, I find preliminary evidence for additional ZFP568 binding partners using an unbiased yeast two-hybrid screen of mouse transcripts expressed at E7. Importantly, TRIM28 was isolated as a binding partner of ZFP568, validating the effectiveness of the screen. I identified numerous novel ZFP568 interaction partners, and categorized high-interest proteins. Specifically, I selected transcriptional regulators because ZFP568 possesses transcriptional repression activity, and cytoskeletal factors because ZFP568 localizes to cytosolic structures within cells.

Based on previous reports indicating that KRAB Zinc Finger proteins interact with SMAD proteins (Jiao et al., 2002), I also carried out directed yeast two-hybrid assays in order to determine whether ZFP568 interacts with any of the SMAD proteins. Interestingly, the KRAB

domain region of ZFP568 interacts with the co-SMAD protein, SMAD4. Together, the results of my interaction studies provide a preliminary collection of ZFP568 interacting proteins besides TRIM28. Further research building on this data may provide validation of the protein-protein interactions, as well as establish the significance of these interactions for ZFP568 functions.

## MATERIALS AND METHODS

### Yeast two-hybrid screening

A yeast two-hybrid screen was carried out as described in Matchmaker GAL4 Two-Hybrid System 3 (Clontech). Briefly, pGBKT7-ZFP568 and pACT-mouse E7.0 library were sequentially transformed into AH109 yeast. Yeast was plated on Ade-, His-, Leu-, Trp- media, and each colony was re-streaked twice on the same media. For DNA extraction, 1 ml of pelleted overnight culture was re-suspended in 500 µl of 1M sorbitol, 0.1M EDTA (pH7.4), and 1 µl/ml BME, and 50 µl of Yeast Lytic Enzyme, and incubated at 30 degrees Celcius for 1 hr. Then, samples were processed as in the bacterial alkaline lysis mini-prep protocol as previously described (Sambrook and Russell, 2001). To identify the library cDNA colonies showing a positive interaction, PCR was performed using pACT vector primers and extension times of 80-240s. Products were treated with Exo-SAP-IT (USB) and sequenced using a 5' vector primer for pACT. Sequencing results were analyzed using nucleotide BLAST against the mouse genome.

### Directed yeast two-hybrid assay

Interactions between ZFP568 and SMAD proteins were tested using protocols from the Matchmaker GAL4 Two-Hybrid System 3 (Clontech). pGBKT7-ZFP568 and pGADT7-SMAD1, SMAD2, SMAD3, SMAD4, SMAD5 SMAD6, and SMAD9 constructs were sequentially co-transformed into AH109 yeast. Co-transformed colonies were re-plated onto Leu-, Trp- X-alpha-gal media for blue/white interaction selection.

**Protein localization by confocal microscopy**

GFP-ZFP568 was transfected into 70% confluent NIH3T3 cells using Lipofectamine 2000 (Invitrogen). Cells were fixed for 15 min in 4% PFA, permealized for 10 min in PBS-0.25% Tween, and treated with TO-PRO (1/1000) for 5 minutes.

**GO analysis**

The complete list of ZFP568-interacting genes was analyzed against a background of all *Mus musculus* genes using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/tools.jsp). Overrepresentation P-values were calculated for gene categories from the GOTERM_BP_ALL database.

**Constructs and primers**

pACT-Mouse E7.0 cDNA library containing $3.5x10^6$ independent transcripts was obtained from Clontech. The library was amplified as described in Matchmaker GAL4 Two-Hybrid System 3 (Clontech). All other constructs were generated as indicated below:

| | |
|---|---|
| pGBKT7-DBD-ZFP568 | EcoRI from PCR-amplified cDNA fragment using primers |
| (full length) | AATTGTCGACACCCAGCCTTGAAATACCAG and |
| | AATTGTCGACTGTTATCCACCACAGGGTTTT |
| pGBKT7-DBD-ZFP568 | EcoRI from PCR-amplified cDNA fragment using primers |
| (KRAB domains) | ATTGTCGACACCCAGCCTTGAAATACCAG and |
| | AATTGTCGACCTCACTGGCCTTTGCCTTAC |
| pGBKT7-DBD-ZFP568 | EcoRI from PCR-amplified cDNA fragment using primers |
| (ZF domains) | AATTGTCGACGTAAGGCAAAGGCCAGTGAG and |
| | AATTGTCGACTGTTATCCACCACAGGGTTTT |
| pAcGFP-ZFP568 | BamHI-XhoI from PCR-amplified cDNA fragment using primers |
| | TCAGATCTCGAGATGGAGCGCTTGTCCCAGATG and |
| | ACCGGTGGATCCCGTTCACTCCTCCGTCCTGTATG |
| pGADT7-TRIM28 | EcoRI from PCR-amplified cDNA fragment using primers |
| | TTAGAATTCTTGCGTGATAGTGGCAGTAAGG and |
| | TAAGAATTCTGGTTCTACCAGCACAGCAG |
| pGADT7-SMAD1 | ClaI/XhoI from PCR-amplified cDNA fragment using primers |
| | TTAGATCGATCTGTGACCAGCTTGTTTTCATTC and |
| | TTATCTCGAGTTCAGAACCTTATCCAGCCACTG |
| pGADT7-SMAD2 | EcoRI from PCR-amplified cDNA fragment using primers |

| | |
|---|---|
| | TTAGAATTCGCAGTGAAAAGTCTGGTGAAAA and<br>TAAGAATTCAAGGGGATCCCATCTGAGTT |
| pGADT7-SMAD3 | EcoRI from PCR-amplified cDNA fragment using primers<br>TTAGAATTCGCCATGTCCATCCTG and<br>TAAGAATTCCATCTGGGTGAGGACCTTGT |
| pGADT7-SMAD4 | EcoRI from PCR-amplified cDNA fragment using primers<br>TTAGAATTCTTAAATACACCAACAAGTAACGATGC and<br>TAAGAATTCGCAGGACTTCATCCAAGAGC |
| pGADT7-SMAD5 | EcoRI from PCR-amplified cDNA fragment using primers<br>TTAGAATTCTTGTCAATGGCCAGCTTGTTTT and<br>TAAGAATTCTGCTTCCCGAGTGCTAGAAT |
| pGADT7-SMAD6 | ClaI/XhoI from PCR-amplified cDNA fragment using primers<br>TTAGATCGATTAGATCCCCAAGCCAGACAGT and<br>TTATCTCGAGCAGCCGCATTGCTATCT |
| pGADT7-SMAD9 | EcoRI from PCR-amplified cDNA fragment using primers<br>TTAGAATTCTTCATCAGCTCCCTCTTCTCCTT<br>TAAGAATTCCCCATCTGAGTGAGCACCTT |

## RESULTS & DISCUSSION

### Unbiased discovery of ZFP568 interacting proteins

In order to uncover novel interaction partners for ZFP568 that are important for its functions during embryonic development, I performed a yeast two-hybrid screen of a mouse

**Table A.1 ZFP568 interaction partners.** List of unique proteins identified as ZFP568 interaction partners from yeast two hybrid screen, sorted from highest to lowest number of clones.

E7.0 cDNA library. We previously found that ZFP568 interacts with TRIM28 in directed yeast two-hybrid and mammalian co-immunoprecipitation experiments (see Chapters 2 and 3) (Shibata et al., 2011). In agreement, I isolated TRIM28 as a binding partner for ZFP568 in the unbiased yeast two-hybrid screen (Table A.1, yellow highlight). The TRIM28 clone that interacted with ZFP568 corresponded to its RBBC domain (data not shown), consistent with reports of TRIM28 interactions with other KRAB Zinc finger proteins (Kim et al., 1996; Moosmann et al., 1996; Ryan et al., 1999). This result validates the effectiveness of the yeast two-hybrid screening methods in identifying genuine ZFP568 interacting proteins.

By screening the E7.0 mouse cDNA library to approximately 40% coverage under high stringency conditions, I identified over 70 unique candidate full-length ZFP568 interacting proteins (Table A.1). In order to differentiate whether different types of proteins were preferentially binding to the KRAB domains or the Zinc Finger domains of ZFP568, I also performed small-scale yeast two hybrid screening experiments using truncated ZFP568 constructs that contained the ZFP568 KRAB domains or the Zinc Finger motifs. Comparison of the interactors of the truncated and full length ZFP568 constructs did not uncover any obvious class of proteins for each domain type (Table A.1). Gene ontology (GO) analysis of all ZFP568 interacting genes revealed that the top overrepresented categories included proteins classified as functioning in “negative regulation of biological processes” and “regulation of localization” (Fig. A.1). Based on the GO analysis, literature searches, and previous data from our laboratory, I categorized high-priority ZFP568 interacting proteins for follow up studies (Table A.2). Because my previous results support a role for ZFP568 as a transcription factor (Chapter 3), I selected yeast two-hybrid ZFP568 interacting proteins that function as transcriptional regulators.

| Symbol | Name | # of clones |
|---|---|---|
| FULL LENGTH | | |
| Flna | filamin, alpha | 16 |
| Cyba | cytochrome b-245, alpha polypeptide | 5 |
| Efemp2 | epidermal growth factor-containing fibulin-like extracellular matrix protein 2 | 5 |
| Gnb2l1 | guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1 | 4 |
| Siva1 | SIVA1, apoptosis-inducing factor | 4 |
| Uxt | ubiquitously expressed transcript | 4 |
| A2m | alpha-2-macroglobulin | 3 |
| Fbln2 | fibulin 2 | 3 |
| Fbxw5 | F-box and WD-40 domain protein 5 | 3 |
| Pcgf1 | polycomb group ring finger 1 | 3 |
| Plac8 | placenta-specific 8 | 3 |
| Aip | aryl-hydrocarbon receptor-interacting protein | 2 |
| Cops3 | COP9 (constitutive photomorphogenic) homolog, subunit 3 (Arabidopsis thaliana) | 2 |
| Cops8 | COP9 (constitutive photomorphogenic) homolog, subunit 8 (Arabidopsis thaliana) | 2 |
| Eftud2 | elongation factor Tu GTP binding domain containing 2 | 2 |
| Fbln5 | fibulin 5 | 2 |
| Pcca | propionyl-Coenzyme A carboxylase, alpha polypeptide | 2 |
| Plcd3 | phospholipase C, delta 3 | 2 |
| Abcf1 | ATP-binding cassette, sub-family F (GCN20), member 1 | 1 |
| actb | actin beta | 1 |
| Alkbh7 | alkB, alkylation repair homolog 7 (E. coli) | 1 |
| Arhgdia | Rho GDP dissociation inhibitor (GDI) alpha | 1 |
| Becn1 | beclin 1, autophagy related | 1 |

| | | |
|---|---|---|
| C1qc | complement component 1, q subcomponent, C chain | 1 |
| Chpf | chondroitin polymerizing factor | 1 |
| Chrd | chordin | 1 |
| Col1a2 | collagen, type I, alpha 2 | 1 |
| Col4a2 | collagen, type IV, alpha 2 | 1 |
| Cryab | crystallin, alpha B | 1 |
| Ddx5 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 | 1 |
| Dynll1 | dynein light chain LC8-type 1 | 1 |
| Egfl7 | EGF-like domain 7 | 1 |
| Fbn1 | fibrillin 1 | 1 |
| Fdxr | ferredoxin reductase | 1 |
| Flnc | filamin C, gamma | 1 |
| Gng12 | guanine nucleotide binding protein (G protein), gamma 12 | 1 |
| Gps1 | G protein pathway suppressor 1 | 1 |
| Gtf2h2 | general transcription factor II H, polypeptide 2 | 1 |
| H19 | H19 fetal liver mRNA | 1 |
| Hmgcs2 | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 | 1 |
| Hnrnpab | heterogeneous nuclear ribonucleoprotein A/B | 1 |
| Hnrnpf | heterogeneous nuclear ribonucleoprotein F | 1 |
| Htra2 | HtrA serine peptidase 2 | 1 |
| Lgals9 | lectin, galactose binding, soluble 9 | 1 |
| LOC100045146 | similar to B cell antigen receptor Ig beta associated protein 1 | 1 |
| LOC633677 | similar to Bifunctional aminoacyl-tRNA synthetase | 1 |
| Marc2 | mitochondrial amidoxime reducing component 2 | 1 |
| Mlf2 | myeloid leukemia factor 2 | 1 |
| Mrpl28 | mitochondrial ribosomal protein L28 | 1 |
| Nid2 | nidogen 2 | 1 |
| Nisch | nischarin | 1 |
| Pfdn5 | prefoldin 5 | 1 |
| Phb2 | prohibitin 2 | 1 |

| | | |
|---|---|---|
| Ppic | peptidylprolyl isomerase C | 1 |
| Ppp2r5b | protein phosphatase 2, regulatory subunit B (B56), beta isoform | 1 |
| Psmb1 | proteasome (prosome, macropain) subunit, beta type 1 | 1 |
| RIKEN0610038F07 | similar to RIKEN cDNA 0610038F07 | 1 |
| RIKEN2310009B15 | RIKEN cDNA 2310009B15 gene | 1 |
| Rmnd5b | required for meiotic nuclear division 5 homolog B (S. cerevisiae) | 1 |
| Sci | short circuit | 1 |
| Sema3f | sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F | 1 |
| Sertad1 | SERTA domain containing 1 | 1 |
| Sfrp5 | secreted frizzled-related sequence protein 5 | 1 |
| Slc25a10 | solute carrier family 25 (mitochondrial carrier, dicarboxylate transporter), member 10 | 1 |
| Slc6a8 | solute carrier family 6 (neurotransmitter transporter, creatine), member 8 | 1 |
| Snapin | SNAP-associated protein | 1 |
| Sphk1 | sphingosine kinase 1 | 1 |
| Srgn | serglycin | 1 |
| Stk4 | serine/threonine kinase 4 | 1 |
| Suox | sulfite oxidase | 1 |
| Tes | testis derived transcript | 1 |
| Tpp1 | tripeptidyl peptidase I | 1 |
| Trim28 | tripartite motif-containing 28 | 1 |
| Troap | trophinin associated protein | 1 |
| Trpc4ap | transient receptor potential cation channel, subfamily C, member 4 associated protein | 1 |
| Wtip | WT1-interacting protein | 1 |
| Yif1a | Yip1 interacting factor homolog A (S. cerevisiae) | 1 |
| Znfx1 | zinc finger, NFX1-type containing 1 | 1 |
| Znrd1 | zinc ribbon domain containing, 1 | 1 |

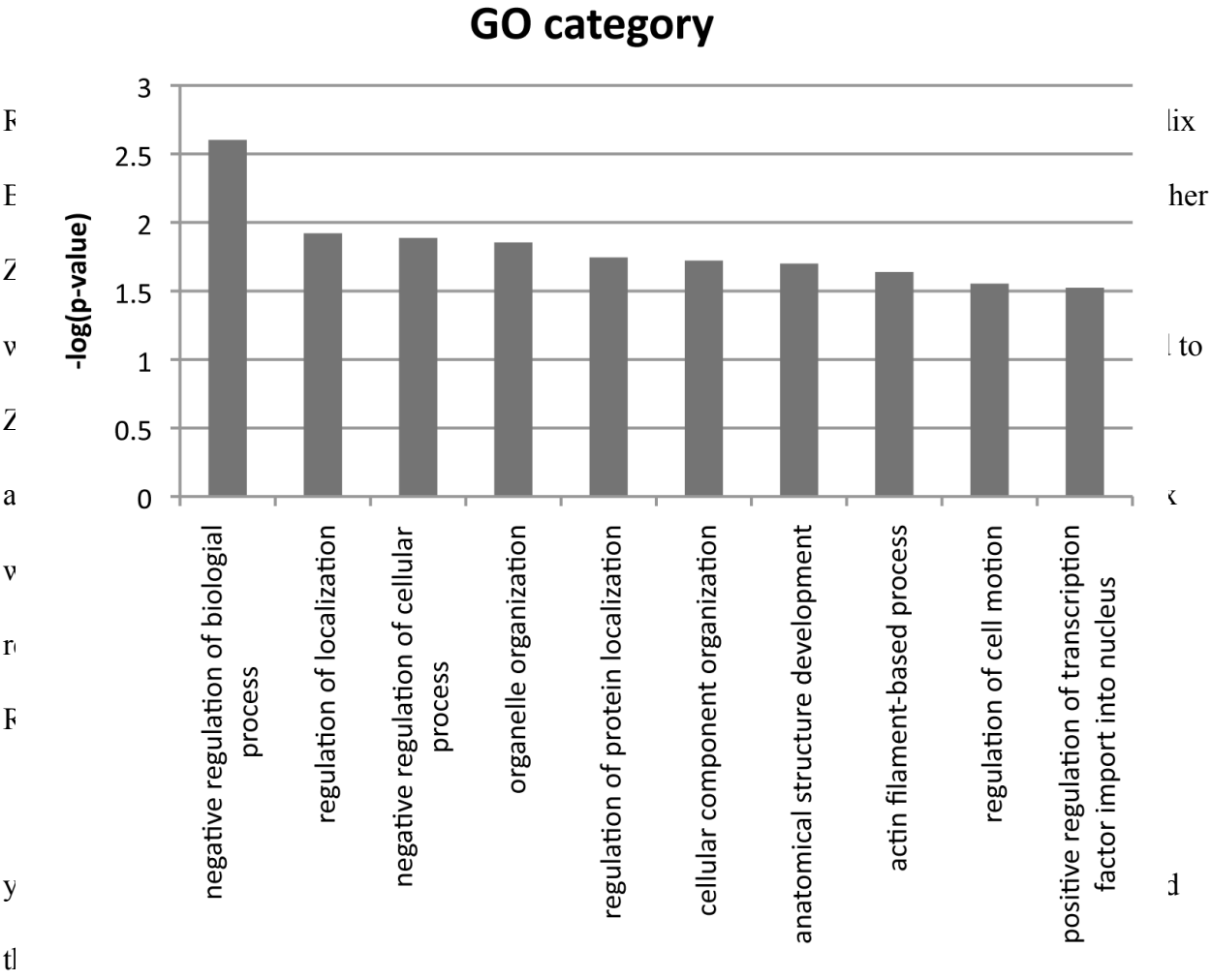
| | | |
|---|---|---|
| KRAB DOMAINS | | |
| Cyba | cytochrome b-245, alpha polypeptide | 2 |
| A2m | alpha-2-macroglobulin | 1 |
| Abp1 | amiloride binding protein 1 (amine oxidase, copper-containing) | 1 |
| Anxa2 | annexin A2 | 1 |
| Bub3 | budding uninhibited by benzimidazoles 3 homolog (S. cerevisiae) | 1 |
| Cd63 | CD63 antigen | 1 |
| Chrd | chordin | 1 |
| Clic1 | chloride intracellular channel 1 | 1 |
| Cryab | crystallin, alpha B | 1 |
| Cybb | cytochrome b-245, beta polypeptide | 1 |
| Dynll1 | dynein light chain LC8-type 1 | 1 |
| Ech1 | enoyl coenzyme A hydratase 1, peroxisomal | 1 |
| Elp2 | elongator acetyltransferase complex subunit 2 | 1 |
| Gnb2l1 | guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1 | 1 |
| Lmo1 | LIM domain only 1 | 1 |
| Mrps28 | mitochondrial ribosomal protein S28 | 1 |
| Ociad1 | OCIA domain containing 1 | 1 |
| Pdcd6ip | programmed cell death 6 interacting protein | 1 |
| Pfdn5 | prefoldin 5 | 1 |
| RIKEN4933407 C03 | RIKEN cDNA 4933407C03 gene | 1 |
| Slc7a5 | solute carrier family 7 (cationic amino acid transporter, y+ system), member 5 | 1 |
| Supv3l1 | suppressor of var1, 3-like 1 (S. cerevisiae) | 1 |
| Tpm1 | tropomyosin 1, alpha | 1 |
| Txn2 | thioredoxin 2 | 1 |
| Ube2i | ubiquitin-conjugating enzyme E2I | 1 |
| Uxt | ubiquitously expressed transcript | 1 |
| Vim | vimentin | 1 |

| | | |
|---|---|---|
| Zfp219 | zinc finger protein 219 | 1 |

ZF DOMAINS

| | | |
|---|---|---|
| RIKEN4930548 G07 | RIKEN cDNA 4930548G07 gene | 2 |
| Cox11 | cytochrome c oxidase assembly protein 11 | 1 |
| Dynlt1b | dynein light chain Tctex-type 1B | 1 |
| Efemp2 | epidermal growth factor-containing fibulin-like extracellular matrix protein 2 | 1 |
| Ewsr1 | Ewing sarcoma breakpoint region 1 | 1 |
| Fbln2 | fibulin 2 | 1 |
| Hnrnpab | heterogeneous nuclear ribonucleoprotein A/B | 1 |
| Lims2 | LIM and senescent cell antigen like domains 2 | 1 |
| Paics | phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoribosylaminoimidazole, succinocarboxamide synthetase | 1 |
| Ptgr1 | prostaglandin reductase 1 | 1 |
| Trip6 | thyroid hormone receptor interactor 6 | 1 |

Additionally, I selected proteins involved in cellular cytoskeletal formation as high-priority candidates based on the sub-cellular cytoplasmic localization of ZFP568 (Chapter 2).

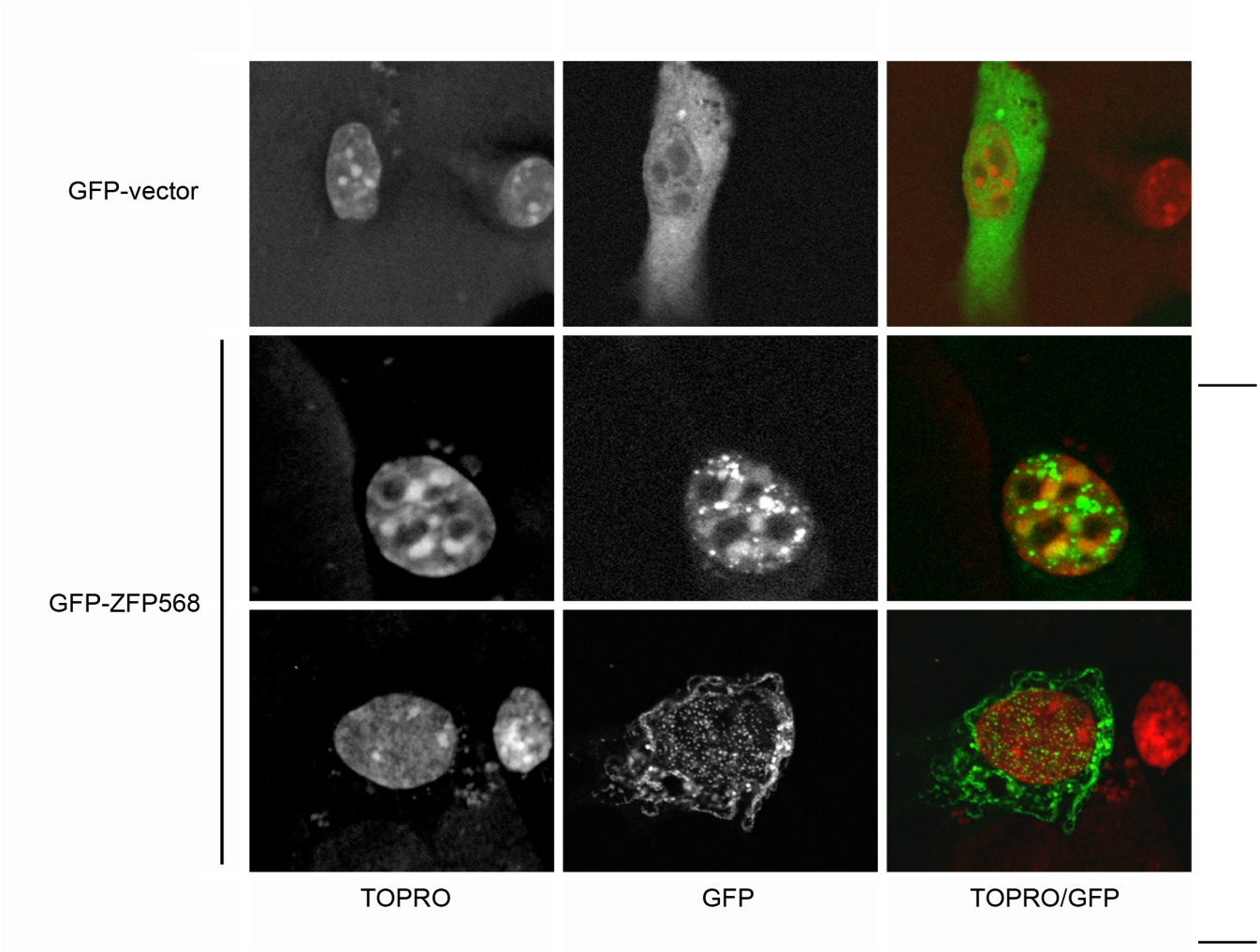



regulation of protein localization, cellular component organization, and actin filament-based process were all overrepresented categories for ZFP568 interacting proteins. Furthermore, I

**Fig. A.1 Gene Ontology (GO) analysis of ZFP568 interacting proteins**. The top 10 overrepresented categories for ZFP568 interacting proteins (p-value) GO analysis was performed using DAVID Bioinformatics Analysis Wizard. LOOK UP GO TERMS

previously observed that ZFP568 localizes to cytoplasmic cellular compartments in mammalian cells (Chapter 2, Fig. A.2). These lines of evidence suggest that cytoskeletal ZFP568-interacting proteins could play an important functional role in regulating ZFP568 sub-cellular localization and/or function. Further experimentation will be required to establish the role of these cytoskeletal in binding ZFP568.

**Table A.2. High priority categories of ZFP568 interacting proteins**. ZFP568 binding partners were categorized as transcriptional regulators and cytoskeletal factors based on GO analysis, literature searches, and data from our laboratory.

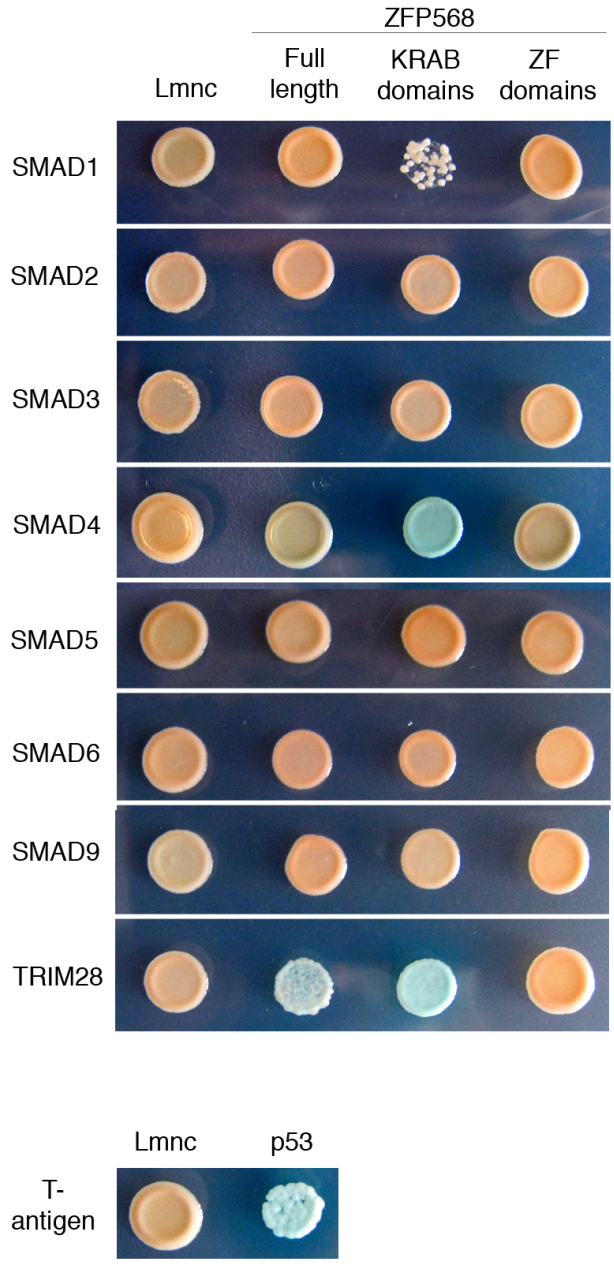



| | |
|---|---|
| Tpm1 | tropomyosin 1, alpha |
| Tcpm1 | T cell phenotype modifier 1 |
| Trip6 | thyroid hormone receptor interactor 6 |
| Flna | filamin, alpha |
| Flnc | filamin C, gamma |
| Dynll1 | dynein light chain LC8-type 1 |
| Dynlt1b | dynein light chain Tctex-type 1B |
| Dbnl | drebrin-like |
| Tes | testis derived transcript |

**Fig. A.2. ZFP568 localizes to specific cytosolic structures.** Subcellular localization of GFP-ZFP568 (green) in two different cells representing different localization patterns observed. Empty GFP vector was used as a control. Samples were costained with TOPRO to mark nucleus.

**Candidate-driven identification of SMAD4 interaction with ZFP568**

The yeast t onditions did not reveal any members of th 568 binding partners. However, previous ger proteins bind SMAD proteins (Jiao et al en ZFP568 and SMAD proteins is illustrat l deletion of mouse SMAD proteins Sl rest and phenotypic similarities to *chai* ang et al., 1999; Tremblay et al., 2001; Chu e l experiments designed to detect weak intera 9. I found that full length ZFP568 did not in er, a truncated construct containing the KR co-SMAD in the Bmp/ TGFß pathway (Fi s will be required to delineate why only )4 by yeast two-hybrid, and what the funct




CONCLUSIONS

Results of the yeast two-hybrid screen identified novel ZFP568 interacting proteins that are potentially critical for ZFP568 functions in transcriptional repression or other processes. Of interest, I identified proteins involved in transcriptional regulation as good candidates for mediating ZFP568 transcriptional functions. Interestingly, two of the proteins that interacted with

**Fig. A.3. KRAB domains of ZFP568 interact with SMAD4.** Yeast two-hybrid assays showed interactin between Gal4DBD-ZFP568 (KRAB domains only) and Gal4AD-SMAD4 as indicated by blue color. Lamin C (LmnC) was used as a negative control. p53 interaction with SV40 large T-antigen was used as a positive control.

ZFP568 also interact with TRIM28. Furthermore, two of the ZFP568 interacting proteins associate with the PRC1 complex. I also identified cytoskeletal factors as ZFP568 interacting proteins. Given that ZFP568 localizes to specific structures within the cytosol, these proteins are of high-priority for follow-up studies to understand the cytoplasmic functions of ZFP568. Lastly, I showed that the KRAB domains of ZFP568 interact with SMAD4. This interaction suggests a possible role for ZFP568 in the SMAD/TGFbeta molecular pathway, which is fundamental to embryonic development. While each ZFP568 interacting protein identified must be investigated with additional functional experiments, the yeast two-hybrid screen provided a framework for important new discoveries about the biological roles of ZFP568.

# APPENDIX B

# ROLES OF PHB2 AS A BINDING PARTNER OF ZFP568

INTRODUCTION

The Polycomb Repressive Complexes (PRC) 1 and 2 are critical for a variety of developmental processes, such as body plan formation and ES cell pluripotency (Richly et al., 2011). The PRC2 functions to repress transcription by catalyzing Histone 3 Lysine 27 trimethylation (H3K27me3), and it has been proposed that PRC1 is recruited to PRC2 to facilitate PRC2-mediated repression. However, the mechanisms by which Polycomb repressive complexes are recruited to DNA are poorly understood in mammals (Simon and Kingston, 2009).

The unbiased yeast two-hybrid screen described in Appendix A of this dissertation identified Prohibitin 2 (PHB2), a PRC1 interacting protein (Lee et al., 2008). Because KRAB Zinc Finger proteins are proposed to recruit transcriptional repressors such as TRIM28 to specific DNA targets, I hypothesized that Polycomb-related protein PHB2 might be functionally significant for ZFP568-mediated repressive activity.

PHB2, like ZFP568, is essential for mouse development prior to E9.0, yet the phenotype of PHB2 null embryos has not been characterized (Park et al., 2005). The early lethality of *Phb2* mutants is attributed to its function in mitochondria respiration (Coates et al., 2001), but PHB2 has also been shown to possesses transcriptional repressive activity, and to recruit chromatin-modifying enzymes such as HDAC1 and PRC1 members to mediate its repression in reporter assays (Kurtev et al., 2004; Lee et al., 2008). Here, I further investigated the interaction between ZFP568 and PHB2. I show that PHB2 co-immunoprecipitates with ZFP568 in mammalian cells.

Furthermore, I provide evidence that PHB2 interacts with TRIM28, raising the possibility that PHB2, TRIM28, and ZFP568 function in a common multi-protein complex. I also show that PHB2 may mediate the repressive activity of ZFP568. These findings suggest that the interaction between ZFP568 and PHB2 could be functionally significant for transcriptional repression.

## MATERIALS AND METHODS

### Co-immunoprecipitation

HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen). For immunoprecipitation, cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1% Triton X-100, 0.05% SDS and protease inhibitors. Immunoprecipitations were performed using 2 µl of anti-PHB2 antibody (Millipore) and 25 µl protein A/G agarose beads (Santa Cruz Biotechnology).

### Sub-Cellular localization

HEK293T cells were transfected with Fugene 6 (Promega). 24 hours after transfection, cells were fixed with 4% PFA, permeabilized with PBS-0.25% Tween, blocked in 1%BSA/PBST, and incubated with anti-PHB2 (1:400) (millipore) and anti-MYC (1:1300) antibodies. Secondary antibodies used were Alexa 488 anti-rabbit (1:10,000) and Alexa 568 anti-mouse (1:10,000). Samples were stained with DAPI (1/4000) for 1 minute.

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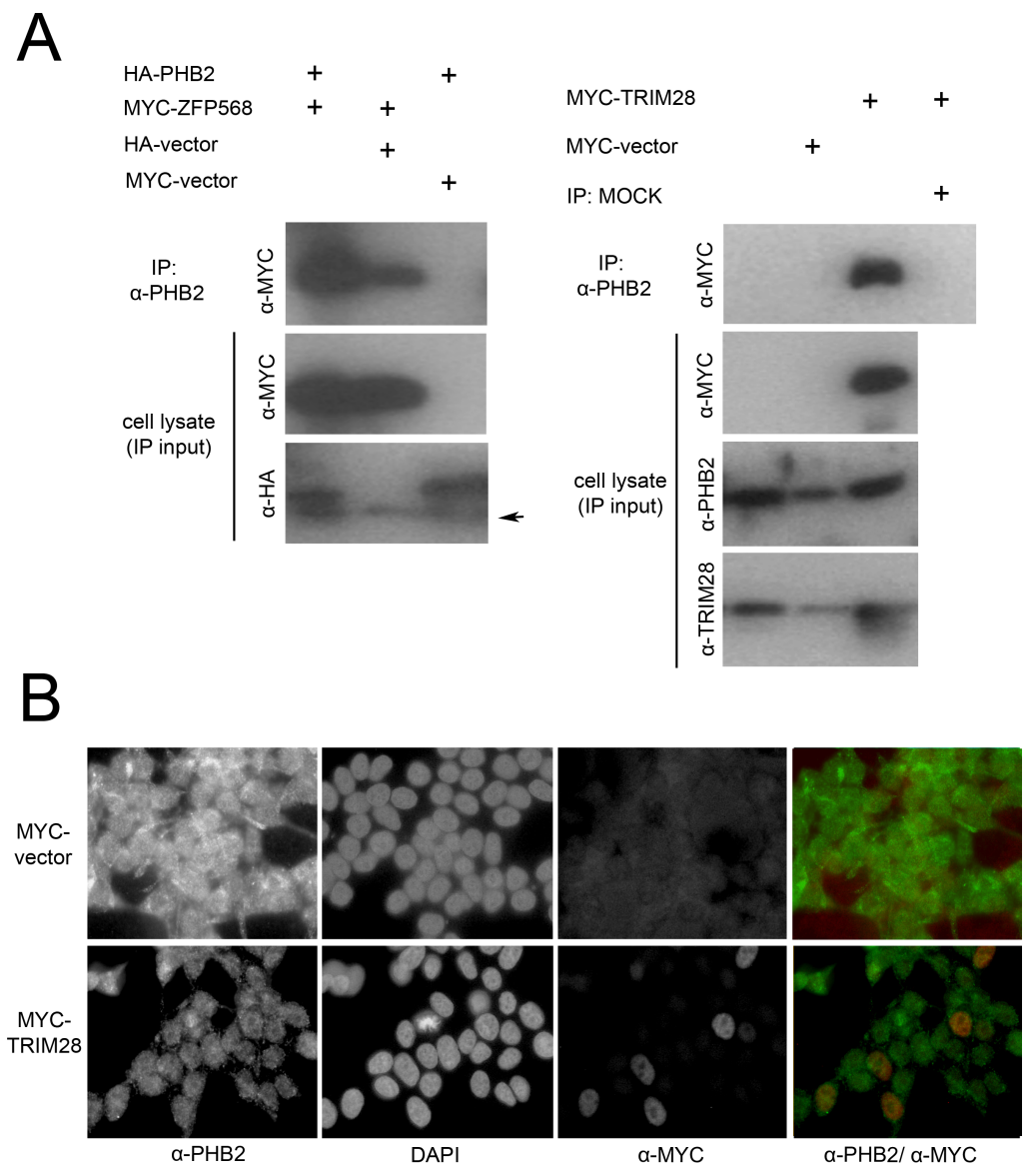


**Fig. B.1 PHB2 interacts with ZFP568 and TRIM28**. (A) PHB2 co-immunoprecipitated (co-IP) with MYC-TRIM28 in transfected HEK293T cells when anti-PHB2 antibody was used to immunoprecipitate. PHB2 also co-immunoprecipitated with MYC-TRIM28 when anti-PHB2 antibody was used to immunoprecipitate (B) Immunohistochemistry with anti-TRIM28 and anti-PHB2 antibodies showing the sub-cellular localization of endogenous PHB2 (green) and transfected MYC-TRIM28 (red) in HEK293T cells. Samples were co-stained with DAPI.

HEK293T cells (Chapter 2). To further understand whether the PHB2 interactions with ZFP568 and TRIM28 occur within the nucleus, I examined the sub-cellular localization of PHB2. Endogenous PHB2 was observed in both nuclear and cytoplasmic compartments of HEK293T cells, but primarily localized to the nucleus (Fig. B.1), in agreement with other reports on PHB2 localization (Kurtev et al., 2004). Moreover, PHB2 co-localized with MYC-TRIM28 within the

nuclear domain of HEK293T cells. Together, the co-immunoprecipitation and sub-cellular localization results suggest that ZFP568, TRIM28, and PHB2 function together within the nucleus. Addi eins interact simul individually.

TRIM sion activity (Moo hibata et al., 2011), a P568 (Chapter 2, Cl e repressive act DBD-ZFP568 prote -PHB2 was overexpre reasing levels of HA- dose-dependent fas 2). This suggests that limited effect on augmenting ZFP568 repressive activity. Additional experiments will be required to understand if high levels of PHB2 titrate out other factors required for repressive activity, such as a PRC1 or PRC2 members.

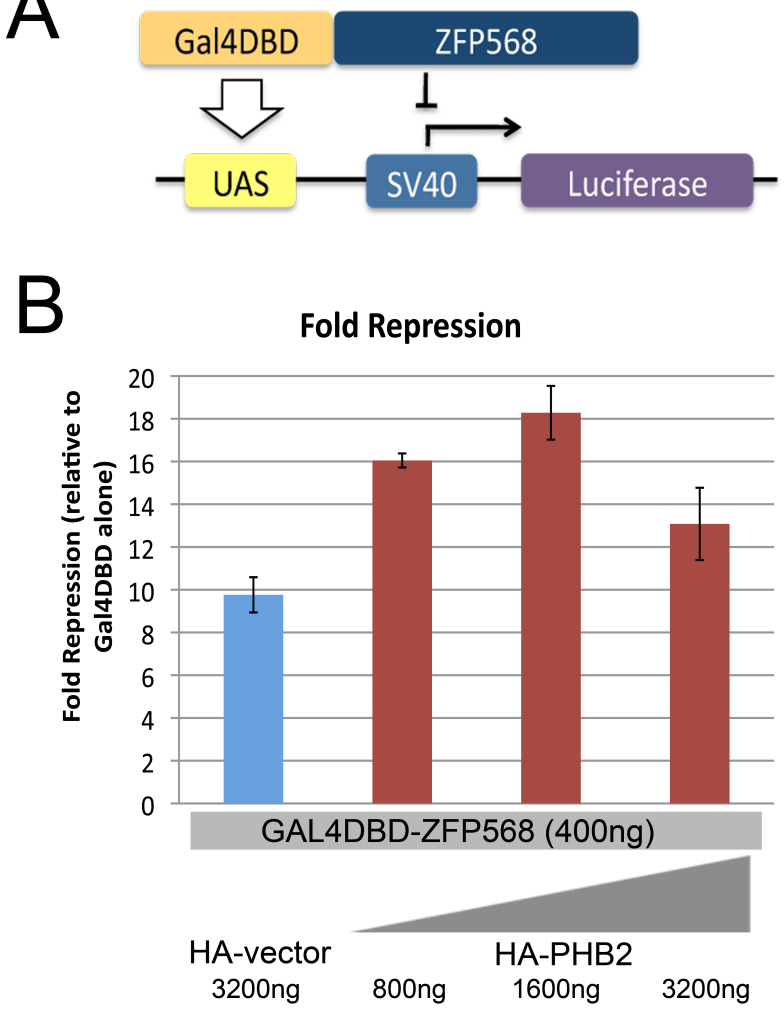


**Fig. B.2. PHB2 mediates ZFP568 repressive activity**. (A) Scheme of the luciferase assay experimental setup illustrating the transfected 5xUAS-luciferase reporter plasmid and the GAL4DBD-ZFP568 effector construct (B) Quantification of luciferase expression in the presence of increasing amounts of HA-PHB2. Luciferase expression is plotted as fold repression relative to Gal4DBD empty vector (1). Error bars represent s.d. Results in B represent one of three experiments showing similar results.

CONCLUSIONS

I verified that the interaction between ZFP568 and PHB2 exists in mammalian cells, and found that PHB2 also interacts with TRIM28 within the nucleus. These interactions represent the first instances of TRIM28 or a KRAB Zinc Finger family member interacting with PHB2. Furthermore, I showed that PHB2 mediates ZFP568 repressive activity, providing evidence that the PHB2-ZFP568 interaction is functionally significant. These results imply that factors other than TRIM28 influence the repressive activities of KRAB Zinc Finger proteins. While these data contribute to the understanding about the functions of ZFP568, follow-up investigations will be necessary in order to determine the mechanism by which PHB2 mediates ZFP568 repressive activity. In addition, future studies aimed to determine the *in vivo* roles of a ZFP568-PHB2 complex must overcome the challenges of separating the two functions of PHB2 in mitochondria metabolism and transcriptional repression.

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